First Evidence for the Presence of a Hydrogenase in the Sulfur-Reducing Bacterium Desulfuromonas acetoxidans

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Received 15 March 1999/Accepted 22 June 1999

Hydrogenases, which are ubiquitous in sulfate-reducing bacteria, were previously thought to be absent from Desulfuromonas acetoxidans. For the first time, a hydrogenase from the strict anaerobic sulfur-respiring bacterium D. acetoxidans, grown on ethanol-malate, was detected and enriched. To assay the role of the hydrogenase in the energetic metabolism of D. acetoxidans, we examined the reactivity of the enzyme with polyheme cytochromes from the same bacterium.

Hydrogenases are enzymes catalyzing both molecular hydrogen production and uptake: \( H_2 \leftrightarrow 2H^+ + 2e^- \). Hydrogen metabolism plays a central role in the energy-generating mechanisms of various microorganisms. For the genus Desulfovibrio, hydrogen cycling with vectorial electron transfer has been proposed as a general energy-coupling mechanism when the cells are grown on organic substrates with sulfate (20). However, since the discovery and description by Pfennig and Biebl of the sulfur reducer Desulfuromonas acetoxidans in 1976, it has been accepted that this specific bacterium lacked hydrogenase activity (24).

D. acetoxidans, a true sulfur-reducing bacterium, is strictly anaerobic, gram negative, flagellated, and rod shaped. It acquires its energy from sulfur respiration and completely oxidizes acetate with \( S^0 \) to carbon dioxide via the citric acid cycle. Reduction of \( S^0 \) produces hydrogen sulfide (H\( _2 \)S). Instead of hydrogenase activity, it was accepted that this specific bacterium lacked hydrogenase activity (24).

D. acetoxidans, grown on ethanol-malate, was detected and enriched. To assay the physiological function of the novel hydrogenase, reactivity with various polyheme D. acetoxidans cytochromes was tested.

D. acetoxidans DSM 1675 was grown at 30°C on malate-ethanol medium containing, per liter, 1 g of KH\( _2 \)PO\( _4 \), 0.3 g of NH\( _4 \)Cl, 1 g of MgSO\( _4 \)· 7H\( _2 \)O, 2 g of MgCl\( _2 \)· 6H\( _2 \)O, 20 g of NaCl, 0.1 g of CaCl\( _2 \)· 2H\( _2 \)O, 1.77 g of Na\( _2 \)SO\( _4 \), 2.6 g of disodium dl-malate, 0.5 g of yeast extract, 1.85 g of NaHCO\( _3 \), 0.3 g of Na\( _2 \)S · 9H\( _2 \)O, 0.3 ml of ethanol, and 10 ml of trace element solution (24). The bacteria were grown on a large scale in a 300-liter anaerobic fermenter, and cells were collected after 18 h (optical density at 600 nm of 0.13). To optimize hydrogenase production, cultures were also grown anaerobically in 20-liter flasks at 30°C in malate-ethanol medium. One-liter aliquots were taken at various times, and the cells were collected by centrifugation (15 min at 5,500 × g).

Except for the extraction step, all purification procedures were performed at pH 7.6 and 4°C with argon-saturated buffers. Hydrogenase activity (hydrogen consumption assay) was measured continually during the purification.

The cells, resuspended in 20 mM Tris-HCl–20 mM NaCl–5 mM EDTA–0.1% Zwittergent 3-12–0.5 mM phenylmethylsulfonyl fluoride, were broken in a French press and centrifuged at 200,000 × g for 1 h. The supernatant was applied to a DEAE-52 column (5 by 20 cm; Whatman) equilibrated with 20 mM Tris-HCl–20 mM NaCl–5 mM EDTA, and a linear gradient of NaCl (20 to 500 mM) in the same buffer was applied. The hydrogenase fraction, eluted between 100 and 200 mM NaCl, was subsequently loaded onto a hydroxylapatite Bio-Gel-HTP (1.5 by 10 cm) column equilibrated in 20 mM Tris-HCl–200 mM NaCl. The hydrogenase fraction was eluted at 200 mM phosphate, dialyzed overnight against 20 mM Tris-HCl, then loaded onto a Q-Sepharose column (fast protein liquid chromatography [FPLC]) equilibrated in the same buffer, and eluted with a gradient of NaCl (0 to 500 mM) (flow rate, 2 ml/min). The column yielded hydrogenase preparation, which was further characterized.

After being activated with sodium dithionite under H\( _2 \), for 20 min, hydrogenase activity was routinely determined at 25°C by the hydrogen consumption assay with methyl viologen as the electron acceptor and hydrogen as the electron donor (9). Hydrogen production from dithionite-reduced methyl viologen was also monitored spectrophotometrically. The anaerobic cuvettes, filled with the oxygen-scavenging system, 50 µM methyl viologen in 50 mM HEPES (pH 7), were flushed for 10 min with argon before addition of 75 µM sodium dithionite. Oxi-
The production of methyl viologen was monitored at 604 nm after the enzyme was added. One unit of hydrogenase activity is the amount of enzyme that catalyzes the reduction of 1 μmol of methyl viologen per min, in the presence of an excess of hydrogen, or the production of 1 μmol of H₂/min.

Cytochrome c₇ and cytochrome c (Mr, 50,000) from D. acetoxidans were purified as previously reported (10, 25). For cytochrome reduction by hydrogenase, anaerobic cuvettes were filled with the oxygen-scavenging system and either cytochrome c₇ or cytochrome c (Mr, 50,000) at a concentration of 5 μM in 50 mM HEPES (pH 7), and the cytochrome reduction rate was measured (9).

Electrophoreses were carried out under native or reducing conditions on a Pharmacia Phast system with 12% polyacrylamide Phast gels and native or sodium dodecyl sulfate (SDS) buffer strips. Hydrogenase activity was revealed on the gel after activation by sodium dithionite (1). After electrophoresis under denaturing conditions, the proteins were transferred onto a nitrocellulose membrane (Pharmacia). Immunodetection was performed with a rabbit anti-Desulfovibrio fructosovorans [NiFe] hydrogenase antiserum (26).

The N-terminal amino acid sequences of the hydrogenase subunits were determined from the enzyme preparation after transfer on a Glassybond membrane. The sequences for the large and the small subunits are RRTAX (data not shown). On the other hand, the [Fe] hydrogenase from Desulfovibrio vulgaris Hildenborough did not cross-react with the same antibodies in the same conditions.

Only one band was observed when the enriched hydrogenase sample was run on native PAGE and revealed with activity stain (Fig. 2A). Two bands of 66 and 27.5 kDa were revealed by SDS-PAGE (Fig. 2B) and Western blotting (Fig. 2C).

The N-terminal sequences of the two subunits were determined after transfer on a Glassybond membrane. The sequences for the large and the small subunits are RRTAX (data not shown). On the other hand, the [Fe] hydrogenase from Desulfovibrio vulgaris Hildenborough did not cross-react with the same antibodies in the same conditions.

The two subunits showed no clear homologies with N-terminal sequences of previously described hydrogenases.

The hydrogenase from D. acetoxidans was active in the assay of H₂ uptake. A methyl viologen reduction by activated enzyme was observed only in the presence of hydrogen. When the enzyme was tested without prior activation, the lag period and

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Protein (mg)</th>
<th>Sp act (U/mg of protein)</th>
<th>Recovery (%)</th>
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</thead>
<tbody>
<tr>
<td>Supernatant of broken cells</td>
<td>32.2</td>
<td>1.218</td>
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<td>0.02</td>
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<td>11</td>
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<tr>
<td>Q-Sepharose (FPLC)</td>
<td>3.6</td>
<td>0.076</td>
<td>46.3</td>
<td>11</td>
</tr>
</tbody>
</table>

X-band spectrometer fitted with an Oxford Instrument He-cryostat and temperature control system.

D. acetoxidans can grow on a medium containing malate as the electron acceptor and ethanol as both carbon and energy sources. Cell yields obtained with this medium are about fourfold higher than with sulfur as the electron acceptor (24). To determine the growth phase yielding the highest levels of hydrogenase, D. acetoxidans was grown anaerobically in 20-liter flasks, and samples were taken at various time intervals. Periplasmic proteins were extracted, and the maximal level of the enzyme was found at the beginning of the exponential phase (Fig. 1). After 24 h of growth, no hydrogenase activity was detected.

The purified enzyme had a UV-visible absorption spectrum typical of an iron-sulfur protein with an A₃₉₀/₄₅₀ of 0.22. The yield was 11% (Table 1).

The D. acetoxidans enzyme cross-reacted with the antibodies raised against the purified [NiFe] hydrogenase from D. fructosivorans (data not shown). On the other hand, the [Fe] hydrogenase from Desulfovibrio vulgaris Hildenborough did not cross-react with the same antibodies in the same conditions.

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the reduction phase appeared considerably longer, indicating reactivation under reducing conditions (at least 60 min for the lag phase). The enzyme was also active in the H₂ evolution assay with dithionite and methyl viologen as donors but at a level about threefold lower, and the lag phase was absent. A slight inhibition of the methyl viologen reduction was observed when 4 μM CO was added to the reaction mixture; 63% of the initial activity remained. [Fe], [NiFe], and [NiFeSe] hydrogenases from D. vulgaris Hildenborough, D. fructosovorans, and D. norvegicum, respectively, were also tested for CO inhibition, and the fractions of residual activity were 10.7, 67, and 85%, respectively.

As described above, only insignificant quantities of hydrogenase were obtained unless the starting material (membrane fraction) was treated with the detergent Zwittergent. Moreover, reactivation under reducing conditions (at least 60 min for the lag phase) was not revealed by the same antibodies. A hydrogenase from D. acetoxidans was detected in the membrane fraction by EPR and Western blotting. The evidenced hydrogenase may be associated with the periplasmic face of the membrane, but we cannot exclude the presence of another membrane-bound hydrogenase.

Several attempts were made to purify the enzyme to homogeneity, but the low detectable amounts and/or the high sensitivity to O₂ precluded an extensive purification. The low amount and stability of the enzyme may explain the recovery loss during the last purification step, which is nonetheless essential.

In Desulfovibrio species, soluble and membrane-bound hydrogenases catalyze the reversible hydrogen oxidation with low-potential multiheme c-type cytochromes, in particular cytochrome c₇ (Mₓ, 13,000). The striking homologies between the tetraheme cytochrome cₓ (Mₓ, 13,000) and the triheme cytochrome cₓ from D. acetoxidans suggest that the two cytochromes may be functionally similar. The cytochrome cₓ was indeed reduced by [NiFe] hydrogenase from D. acetoxidans. In vitro, in the presence of D. vulgaris Hildenborough [Fe] hydrogenase, the cytochrome cₓ has a polysulfide reductase activity (22). It has been proposed that this cytochrome is the terminal reductase, although the physiological electron donor remains to be identified. The hydrogenase that we characterized may be the physiological electron donor for the cytochrome. In addition to cytochrome cₓ, the 50-kDa cytochrome c and the 65-kDa cytochrome c (10, 22) are present in this bacterium. Under our conditions, the reduction of the 50-kDa cytochrome c by the hydrogenase from D. acetoxidans is very inefficient in comparison to the reduction of cytochrome cₓ, and indicates another role for the cytochrome. Further studies will determine whether the cytochrome c (Mₓ, 10,000), the cytochrome c (Mₓ, 50,000), and the cytochrome c (Mₓ, 65,000) could be physiological partners of hydrogenases. In the case of the sulfate-reducing bacteria, the role in D. acetoxidans of several c-type multiheme cytochromes with low redox potential is not understood.

Nonetheless, numerous bacteria with a hydrogenase activity do not possess multiheme cytochromes and can reduce other redox proteins. Multiheme cytochromes as obligatory electron acceptors from hydrogenases are specific to the genus Desulfovibrio. We thus hesitate to exclude electron acceptors other than the multiheme cytochromes for electrons resulting from oxidation of molecular hydrogen by hydrogenase from D. acetoxidans. There are several examples of electron transfer from a membrane-associated hydrogenase to a b-type cytochrome (8, 12, 13, 16). The sulfur-reducing microaerophilic bacterium Spirillum strain 5175 can grow anaerobically with sulfur as the electron acceptor as D. acetoxidans and several Campylobacter species do, and it contains two cytochromes b and a [NiFe] hydrogenase in the membrane (29). These bacteria do not possess multiheme cytochromes. It has been concluded that the b-type cytochromes are involved in the electron transfer from hydrogenase to H₂S. Under certain growth conditions, the presence of a menaquinone and cytochrome b has been validated by electron spin resonance (ESR) spectroscopy and 1H NMR spectroscopy. The hydrogenase from D. acetoxidans was detected in the membrane fraction by EPR and Western blotting. The evidenced hydrogenase may be associated with the periplasmic face of the membrane, but we cannot exclude the presence of another membrane-bound hydrogenase.

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demonstrated in *D. acetoxidans* (14, 21, 24). We have not detected any b-type subunit during purification of the hydrogenase, but experiments are in progress in our laboratory to determine whether the hydrogenase from *D. acetoxidans* reduces endogenous cytochrome b and to clone the *D. acetoxidans* hydrogenase gene. The cloning will indicate whether the hydrogenase gene is in an operon with the gene of a b-type cytochrome, as is the case for several membrane-bound [NiFe] hydrogenases. Further physiological, genetic, and structural studies on the multiheme cytochromes c, the b-type cytochrome(s), and the hydrogenase are required to elucidate the electron transport pathways involving the hydrogenase and its role in the metabolism of this sulfur-reducing bacterium, which had been previously described as not using H₂ as an electron donor.

We thank G. Leroy for technical assistance, Z. Dermoun for the production of antibodies, A. Dolla for helpful discussion and S. Wells for reading the manuscript.

REFERENCES

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