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Characterization of the [NiFe] Hydrogenase from the Sulfate Reducer *Desulfovibrio vulgaris* Hildenborough

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The [NiFe] hydrogenase from *Desulfovibrio vulgaris* Hildenborough was isolated from the cytoplasmic membranes and characterized by EPR spectroscopy. It has a total molecular mass of 98.7 kDa (subunits of 66.4 and 32.3 kDa), and contains 1 nickel and 12 Fe atoms per heterodimer. The catalytic activities for hydrogen consumption and production were determined to be 174 and 89 μmol $H_2 \cdot min^{-1} \cdot mg^{-1}$, respectively. As isolated, under aerobic conditions, this hydrogenase exhibits EPR signals characteristic of the nickel centers in [NiFe] hydrogenases (Ni-A signal at $g_{x,y,z}$=2.32, 2.23 and ~2.0 and Ni-B signal at $g_{x,y,z}$=2.33, 2.16 and ~2.0) as well as an intense quasi-isotropic signal centered at g=2.02 due to the oxidized [3Fe-4S] center. The redox profile under hydrogen atmosphere is remarkably similar to that of other [NiFe] hydrogenases. The signals observed for the oxidized state disappear, first being substituted by the Ni-C type signal ($g_{x,y,z}$=2.19, 2.14, ~2.01), which upon long incubation under hydrogen yields the split Ni-C signal due to interaction with the reduced [4Fe-4S] centers.

Three different kinds of hydrogenases have been characterized from various species of *Desulfovibrio* (*D.*), which can be distinguished in terms of their metal contents, type of redox centers, amino acid sequences, sensitivities to inhibitors and immunological reactivities: the Fe-only ([Fe]) hydrogenases, the nickel-iron ([NiFe]) hydrogenases and the nickel-iron-selenium ([NiFeSe]) hydrogenases (1). The [NiFe] are the most common type of hydrogenases being found in bacteria as well as in archaea (2).

In *Desulfovibrio* it has been found that the gene for the [NiFe] hydrogenase is present in all of the screened species, whereas the genes for the [Fe] and [NiFeSe] enzymes have been found in only some of them (3). The presence of distinct hydrogenases in the same bacterium, with different cellular localizations, is yet poorly understood. It may reflect a mechanism for metabolic regulation, which might depend on the growth conditions. For example, the requirement of two hydrogenases for the reduction of sulfate to sulfite may be fundamental in situations where sulfate is limiting (4,5). The presence of multiple hydrogenases is a key feature of the hydrogen-cycling hypothesis, a proposed bioenergetic mechanism in *Desulfovibrio* in which molecular hydrogen is involved in the generation of a proton motive force (4).

D. vulgaris Hildenborough (*Dv*H) contains the genes for the three different hydrogenases (3). The periplasmic [Fe] hydrogenase was the first to be purified and has been extensively characterized (1,6). The presence of [NiFe] and [NiFeSe] hydrogenases in the membranes of *Dv*H was later described (7,8), and the enzymes were partially purified (7). The membrane localization of the two hydrogenases was confirmed by immunoelectron microscopic studies (5). They have different orientations, with the [NiFe] hydrogenase facing the periplasmic space, and the [NiFeSe] hydrogenase oriented towards the cytoplasm. This unique spatial orientation is in agreement with the requirements for the hydrogen cycling hypothesis.

Most of the [NiFe] hydrogenases isolated from *Desulfovibrio* have a periplasmic location, with the exception of the membrane-bound enzymes from *Dv*H and from *D.vulgaris* Miyazaki F (9). This is in contrast with the case of the [NiFe] hydrogenases from most other bacteria like *Escherichia coli, Bradyrhizobium japonicum, Rhodobacter capsulatus, Azotobacter vinelandii, Wolinella succinogenes* and others, which are all membrane-bound (10). These last enzymes are encoded by polycistronic operons, and have a hydrophobic sequence of about 50 amino acids in the COOH-terminus of the small subunit, which possibly serves as a membrane anchor (11). The *Desulfovibrio* enzymes are encoded by simple, bicistronic operons, and even the two hydrogenases purified from the membranes do not have the hydrophobic COOH-terminus in the small subunit (10,11).

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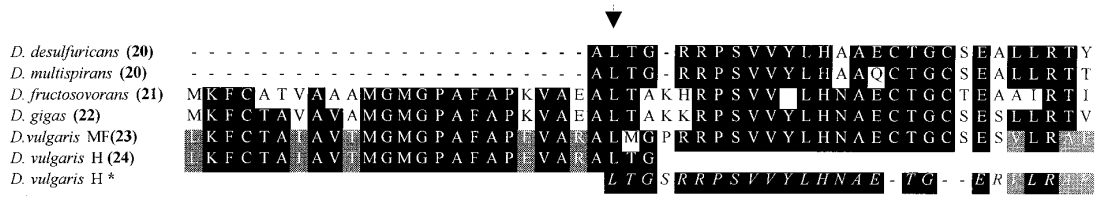

FIG. 1. Comparison of the N-terminal sequences of *Desulfovibrio* [NiFe] hydrogenases small subunits. The arrow points the beginning of the N-terminal sequences. * Sequence found for the hydrogenase purified in this work.

In this paper we describe the full purification and spectroscopic characterization of the *D.vulgaris* membrane-bound [NiFe] hydrogenase, which was preliminary described previously (7).

MATERIALS AND METHODS

Cell growth and preparation of the membrane extract. *Desulfovibrio vulgaris* Hildenborough (ATCC 29579) was grown in lactate/sulfate medium as previously described (12). The preparation of the membrane extract was performed as in (13). The membranes were repeatedly washed with Tris-HCl buffer 10mM pH 7.6 to remove soluble and weakly bound proteins, suspended in 50mM Tris-HCl buffer pH7.6, and then solubilized by addition of 2% SB12™ (w/v) (N-dodecyl-N,N-dimethyl-3-ammonio-1-propansulfonate). The membrane extract was dialized against 0.2% SB12 in 20mM Tris-HCl buffer pH 7.6.

Protein purification. All purification procedures were performed at pH 7.6 and 4°C, and in the presence of 0.2% (w/v) SB12. The dialized extract was loaded on a DEAE-52 column (Whatman, 6×40cm) equilibrated with 20mM Tris-HCl buffer, and a linear gradient of 50mM-400mM NaCl (21) in the same buffer was applied; two fractions with hydrogenase activity were eluted at 200mM NaCl and 300mM NaCl. The first fraction was further purified through passage on a Pharmacia Q-Sepharose (XK26/20) column (2ml/min) equilibrated with 20mM Tris-HCl, and eluted with a stepwise gradient of NaCl. One fraction with hydrogenase activity was eluted at 300mM NaCl. This fraction was applied to a hydroxyapatite column (Bio-Rad, 1.5×15cm) equilibrated with 5mM potassium phosphate buffer and eluted with a linear gradient of 5-300 mM phosphate buffer. The hydrogenase containing fraction was then passed on a Superdex 200 column (Pharmacia XK16/40) equilibrated and eluted with 100mM Tris-HCl, 100mM NaCl (2ml/min). The hydrogenase fraction was finally purified on a Pharmacia Q-Sepharose column (XK26/20) equilibrated with 20mM Tris-HCl, and eluted with a stepwise gradient of NaCl. This column yielded pure [NiFe] hydrogenase as judged by EPR and SDS-PAGE.

Analytical methods. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (14). The protein molecular masses were determined by 12% SDS-PAGE using Pharmacia low-range protein standards. The concentration of hydrogenase was determined from the absorbance at 400 nm using an absorption coefficient of 46.5 $mM^{-1}cm^{-1}$ (15). The N-terminal sequence was determined by the method of Edman and Begg (16) using an Applied Biosystem model 470A sequenator. Metal contents were determined by atomic absorption on a graphite chamber.

Enzymatic measurements. Hydrogenase activity in hydrogen production was determined by Gas-chromatography, at pH 7.0 as described in (17); hydrogen consumption activity was measured spectrophotometrically at 25°C in anaerobic cuvettes using benzyl viologen as the electron acceptor (18). Inhibition by nitrite was tested with the hydrogen consumption assay, by adding an anaerobic solution of nitrite to an actively H_2-consuming reaction mixture. The concentration of nitrite in the reaction mixture was 0.5mM.

Spectroscopic methods. UV-Visible spectra were obtained on a Shimadzu UV 1603 spectrophotometer. EPR spectra were recorded using a Bruker ESP 380 spectrometer equipped with continuous-flow cryostats for liquid helium or liquid nitrogen.

RESULTS AND DISCUSSION

Two fractions with hydrogenase activity were obtained after the first chromatographic step. The first of these fractions contained the [NiFe] hydrogenase, which was purified to almost complete homogeneity. This enzyme has two subunits with apparent molecular masses of 66.4 and 32.3 kDa, determined by SDS-PAGE. It contains one nickel atom and 12±1 iron atoms per heterodimer; selenium was not detected. The hydrogen production activity using methyl viologen as electron donor was determined to be 174.3 μmol $H_2 \cdot min^{-1} \cdot mg^{-1}$; hydrogen uptake activity with benzyl viologen as the electron acceptor was found to be 89.0 μmol $H_2 \cdot min^{-1} \cdot mg^{-1}$. The hydrogen-uptake activity was not inhibited by nitrite, in agreement to what is found for other [NiFe] hydrogenases (19).

The N-terminal sequence of the small subunit of the purified hydrogenase shows a high degree of homology with those of the other *Desulfovibrio* [NiFe] hydrogenases (Figure 1). The highest homology is found with the N-terminal of the [NiFe] hydrogenase small subunit from *D. vulgaris* Miyazaki F, followed by that of *D. gigas*. This is in agreement with the phylogenetic tree constructed by Wu & Mandrand (10) based on the gene sequence of these hydrogenases, which shows that the *D. vulgaris* Miyazaki F and Hildenborough [NiFe] hydrogenases are the two more closely related, followed closely by the *D. gigas* and *D. fructosovorans* hydrogenases.

The ultraviolet-visible spectrum is characteristic of iron-sulfur proteins, showing a broad absorption band centered at 400nm (Figure 2), and with a A_{400}/A_{280} ratio of 0.23, within the range of other [NiFe] hydrogenases.

In the oxidized form the EPR spectrum is dominated by an almost isotropic signal ($g_{x,y,z}$=2.026, 2.018, 2.012) due to the $[3Fe\text{-}4S]^{1+}$ center (Figures 3A, spectrum i and 3B, spectrum i). Ni-A and Ni-B type signals are also detected (Figure 3A, spectrum i), with $g_{x,y}$ values at 2.320, 2.235 and at 2.330, 2.160, respectively. These two

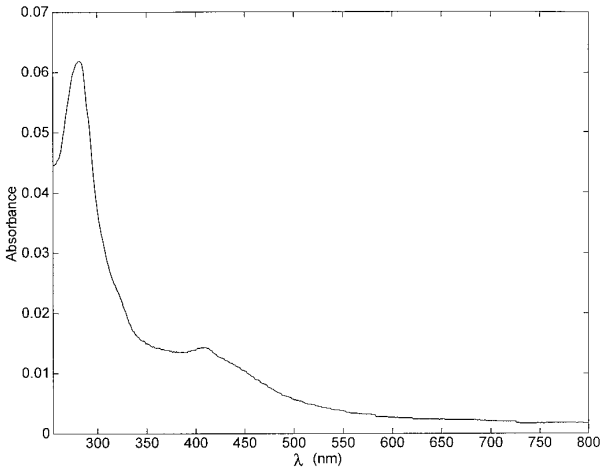

FIG. 2. UV–Visible spectrum of the purified [NiFe] hydrogenase of *Dv*H.

species are present in a 1:1 ratio, as deduced by theoretically simulating both nickel components (Table 1). Using these simulations, it is observed that the sum of the intensities of the two nickel signals is stoichiometric with that of the $[3Fe\text{-}4S]^{1+}$ center signal. A minor component with a distinct g-value at ~2.26 (Figure 3A, spectrum i) is also observed, which in the case of *D.vulgaris* Miyazaki F (25) and *Chromatium vinosum* (26) [NiFe] hydrogenases was tentatively attributed to a coupling between the nickel center and another paramagnet, possibly the $[3Fe\text{-}4S]^{1+}$ cluster. After reduction with hydrogen (Figure 3A, spectrum ii) and reoxidation of the *Dv*H hydrogenase this species is no longer detected, and the intensity of the Ni-A signal increases in relation to the Ni-B resonances (Figure 3A, spectrum iii).

Upon exposure to hydrogen an EPR "silent state" is achieved, in which the signal of the reduced [3Fe-4S] center is well observed (Figure 3B, spectrum ii). After prolonged incubation with molecular hydrogen, the low field resonance of the [3Fe-4S] center changes due to the magnetic interaction with the $[4Fe\text{-}4S]^{1+}$ center(s), with a very broad signal appearing, Fe-S signal B (27), (Figure 3B, spectrum iii). Concomitantly the [4Fe-4S] centers are reduced and another broad signal appears, Fe-S signal B′ (27), (Figure 3B, spectrum iii). Superimposed with the Fe-S signal B′, the "g=2.22" type signal (or split Ni-C signal) (27) (Figure 3A, spectrum ii), is clearly detected with main features at g=2.22, 2.20, 2.18 and 2.10; at liquid nitrogen temperature the Ni-C signal ($g_{x,y,z}$=2.19, 2.15, 2.01) was observed. A summary of the EPR signals detected is presented in Table 1.

The overall spectral profile is remarkably similar to that of other [NiFe] hydrogenases, in particular to that of the *D.gigas* (27) and *D.vulgaris* Miyazaki F (28) enzymes, which strongly suggests that the overall structure of the metal centers is very similar in the soluble and membrane-bound hydrogenases.

CONCLUSIONS

This report details the purification and characterization of the membrane-bound [NiFe] hydrogenase from *Dv*H. The data presented shows that in spite of the different cellular localizations, the soluble and membrane-bound [NiFe] hydrogenases from *Desulfovibrio* species are very similar, in terms of subunit composition, activity and architecture of the metal centers. The membrane-bound [NiFe] hydrogenase from *Dv*H is indeed very similar to the enzyme from *D.gigas* and *D.vulgaris* Miyazaki F, containing one $[3Fe\text{-}4S]^{1+/0}$ and two $[4Fe\text{-}4S]^{2+/1+}$ centers, plus the nickel center which, due to the similarity of the EPR redox profile is most probably also a bimetallic Ni-Fe center (29).

The similarity between the periplasmic and membrane-bound *Desulfovibrio* [NiFe] hydrogenases, and

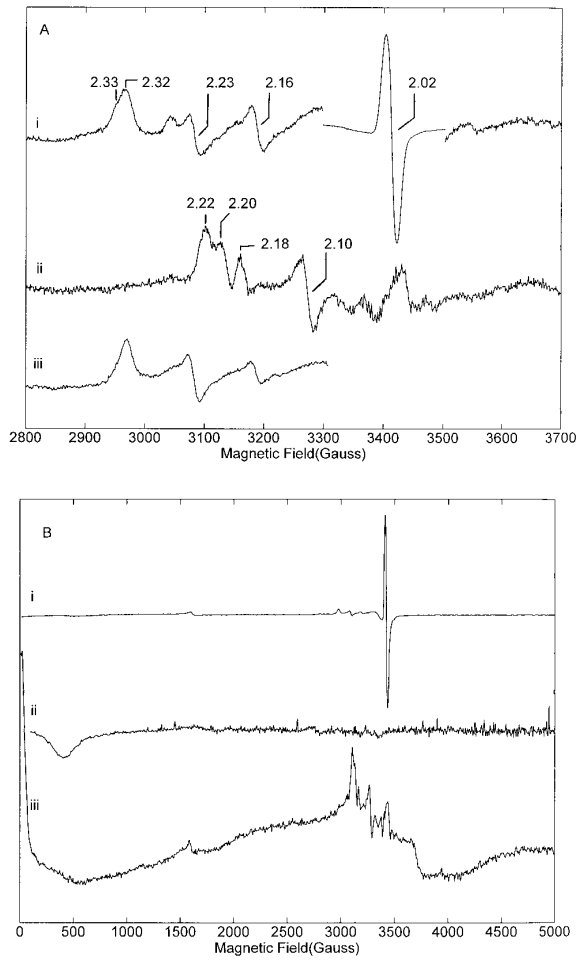

FIG. 3. EPR spectra of the purified *D. vulgaris* [NiFe] hydrogenase (A) (i) as isolated (the Ni signals are multiplied 16 times in relation to the 3Fe signal), (ii) reduced under hydrogen, (iii) reoxidized; (b) (i) as isolated, (ii) reduced under molecular hydrogen (parallel mode EPR), (iii) further reduced. Temperature, 4.7 K; microwave frequency, 9.64 GHz (normal mode); 9.39 GHz (parallel mode); microwave power, 2.4 mW. Variable gain.

TABLE 1

EPR Spectra Detected in *D. vulgaris* Hildenborough [NiFe] Hydrogenase (Nomenclature as in 27))

	Oxidized	Reduced
Nickel	Ni-A 2.320, 2.235, ~2.02(*)	Ni-C: 2.19, 2.15, 2.01
	Ni-B 2.330, 2.160, ~2.02(*)	Split Ni-C: 2.22, 2.20, 2.18, 2.10
Fe-S centers	$[3Fe\text{-}4S]^{1+}$ 2.026, 2.018, 2.012	$[3Fe\text{-}4S]^{0}$ (through at g ~ 16)
		$[3Fe\text{-}4S]^{0}$ Fe-S signal B (through at g ~ 13)
		$[4Fe\text{-}4S]^{1+}$ Fe-S signal B′

Note. For the oxidized form the g values presented were obtained by simulation of the experimental spectra. (*) not detected due to superposition with the $[3Fe\text{-}4S]^{1+}$ signal.

the fact that neither have a hydrophobic membrane-anchor raises doubts about the true membrane-bound nature of the *D.vulgaris* Hildenborough and Miyazaki enzymes. We have observed that the *Dv*H enzyme could not be removed from the membranes by washing with low-ionic strength buffer, and that it could not be purified in the absence of detergent, which led to precipitation of the protein. So, there is most probably some sort of membrane-association in the case of the Hildenborough and Miyazaki enzymes, that is absent or is less pronounced in the case of the other hydrogenases purified from the periplasm. This association is possibly not a direct association with the membrane phospholipids, as the hydrogenases are not very hydrophobic, but may exist via a membrane-bound protein complex. A membrane association was also observed in the localization studies performed in *Dv*H (5). The physiological significance of this membrane-association is not obvious, and it is interesting to note that in *D.vulgaris* Miyazaki F only the [NiFe] and [NiFeSe] hydrogenases were detected, and not the highly active periplasmic [Fe] hydrogenase (3), so that its metabolism probably operates in a different way from *Dv*H.

An operon coding for a transmembrane protein complex has been sequenced in *Dv*H (30), which contains the high-molecular mass, sixteen-heme cytochrome (Hmc). As in the case of the [NiFe] hydrogenase, this cytochrome is not hydrophobic but is membrane-associated (13). Recent studies (31) have shown the existence of a fine-tuned mechanism in *Dv*H regulating the amounts of Hmc complex and lactate dehydrogenase present, in relation to the available electron donor. When the cells are grown with hydrogen as electron donor, expression of the Hmc operon is about three times higher than when they are grown with lactate. In addition, a mutant lacking regulatory genes for the Hmc operon overproduced it, and this overproduction induced a decrease in growth rate when compared to the wild type for cells grown with lactate, but an increase for cells grown with hydrogen (31). The domain nature of Hmc has lead to the suggestion that in *Dv*H each domain of Hmc interacts specifically with each of the three hydrogenases (30). However, preliminary studies (13) have showed that this cytochrome is only very slowly reduced in the presence of large amounts of the [Fe] hydrogenase. Also, Hmc is present in strains, like *D.vulgaris* Miyazaki F (32) and *D.gigas* (33), where the [Fe] where the [Fe] hydrogenase or its gene (3) could never be detected. Altogether, these observations suggest that the membrane-associated Ni-containing hydrogenases are the ones preferentially involved in utilization of hydrogen as electron donor, and in interaction with Hmc.

The role of the [Fe] hydrogenase is less clear. It has been shown that both its production and activity are controlled by iron concentration, since cells grown with a limited amount of iron contained 100 times less [Fe] hydrogenase activity than bacteria grown with normal iron concentrations (34). However, both cells grew equally well on a lactate/sulfate medium. These results are in sharp contrast with others that indicate that reduction of the amount of periplasmic [Fe] hydrogenase using antisense RNA, severely affected growth on lactate/sulfate (35). Also, it is to be noted that, although *D.vulgaris* Miyazaki F and *D. gigas* are devoid of the [Fe] hydrogenase gene, they grow equally well on lactate/sulfate, so that, at the present time, its role cannot be ascertained. The purification of the third *Dv*H enzyme, the [NiFeSe] hydrogenase, is under way and will be reported later.

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