Evidence for Nickel and a Three-iron Center in the Hydrogenase of Desulfovibrio desulfuricans

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Hydrogenase from Desulfovibrio desulfuricans (ATCC No. 27774) grown in unenriched and in enriched 65Ni and 57Fe media has been purified to apparent homogeneity. Two fractions of enzymes with hydrogenase activity were separated and were termed hydrogenase I and hydrogenase II. They were shown to have similar molecular weights (77,600 for hydrogenase I and 75,500 for hydrogenase II), to be composed of two polypeptide chains, and to contain Ni and non-heme iron. Because of its higher specific activity (152 versus 97) hydrogenase II was selected for EPR and Mossbauer studies.

As isolated, hydrogenase II exhibits an "isotropic" EPR signal at g = 2.02 and a rhombic EPR signal at g = 2.3, 2.2, and 2.0. Isotopic substitution of 65Ni proves that the rhombic signal is due to Ni. Combining the Mossbauer and EPR data, the isotropic g = 2.02 EPR signal was shown to originate from a 3Fe cluster which may have oxygenous or nitrogenous ligands. In addition, the Mossbauer data also revealed two [4Fe-4S] clusters in each molecule of hydrogenase II. The EPR and Mossbauer data of hydrogenase I were found to be identical to those of hydrogenase II, indicating that both enzymes have common metallic centers.

Hydrogenases have been purified to apparent homogeneity from a number of microbial species and found to exhibit a diversity (1) which is unexpected in view of the simplicity of the reaction involved in the activation of hydrogen. Generally, hydrogenases contain 4 to 12 atoms of non-heme iron/molecule. EPR studies suggest that the types of non-heme iron clusters may have considerable variability (1-3). Most recently, nickel has been shown to be required for the biosynthesis of hydrogenase (1, 4-7) and reported to be a structural component of the hydrogenases from Desulfovibrio gigas (2, 8) and Methanobacterium thermotogotrophicum (5). Both hydrogenases were demonstrated to contain 1 atom of nickel/molecule; however, only the hydrogenase from D. gigas was demonstrated to contain EPR-active non-heme iron. In the oxidized form (i.e. as isolated), both enzymes exhibit EPR signals with g values of 2.3, 2.2, and 2.0 (2, 8). Using isotopic substitution of 65Ni, the EPR signal of Methanobacterium thermotogotrophicum hydrogenase was proven to have arisen from Ni (9). Based on the EPR spectrum originating from Ni(II) in the membranes of M. bryantii (10, 11), the EPR signal from D. gigas hydrogenase was also proposed to reflect the presence of Ni(III), but in contrast to the hydrogenase from M. thermotogotrophicum, reduced D. gigas hydrogenase exhibited Ni signals. Similar g = 2.3 EPR signals have been detected in a high molecular complex containing hydrogenase from M. thermotogotrophicum (12).

In this communication, we describe the purification and characterization of a hydrogenase from cells of Desulfovibrio desulfuricans grown in unenriched and enriched 65Ni and 57Fe media. EPR signals with g values at 2.32, 2.21 and 2.01 have been unequivocally shown to be due to Ni, and Mossbauer studies indicate the presence of two [4Fe-4S] clusters plus a 3Fe cluster.

**MATERIALS AND METHODS**

**Growth of Microorganism and Preparation of Extract—** D. desulfuricans (ATCC No. 27774) was grown in the medium described by Liu and Peck (13) containing nitrate rather than sulfate as a terminal electron acceptor thus avoiding precipitation of metal sulfides. For the growth of isotopically labeled cells, 40 mg of 65Ni (enrichment 86.4%) and 400 mg of 57Fe (enrichment 95%) were first dissolved in H2SO4, then in HC1, neutralized, and added to 400 ml of media. The 65Ni was obtained from Oak Ridge National Laboratory and the 57Fe from New England Nuclear. In a typical preparation of the crude extract, 350 g of cells were suspended in 1 liter of 10 mM Tris-HCl, pH 7.6, and ruptured in a French press at 9,000 p.s.i. under a N2 atmosphere. The extract was centrifuged at 19,000 × g for 30 min and then at 180,000 × g for 75 min.

**Assays—** Hydrogenase activity was determined by the H2 evolution assay (14). Hydrogen was determined by means of a Varian 4000 gas chromatograph (2); iron by the method of Van De Bogart and Beinert (15); and sulfide by the method of Siegel (16). Nickel was determined by plasma emission spectroscopy using the Jarrell-Ash model 560 Atomicomp. Protein was determined by the Bradford method (17) using bovine serum albumin as standard. The Bradford method was chosen because it gave values which were close to the ones obtained from calculations based on the extinction coefficient for D. gigas hydrogenase which has similar chromophore content.

**Electrophoresis—** Purity of the hydrogenase was established by polyacrylamide disc electrophoresis (18) as well as by sodium dodecyl sulfate-polyacrylamide electrophoresis (19).

**Purification of Hydrogenase—** For all purification procedures, the temperature was maintained at 5 °C and the pH of the buffers was 7.6 (measured at 5 °C for Tris-HCl). Precautions were taken against oxygen by flushing buffers with purified argon.

**First DEA-Bio-Gel Column—** A DEA-Bio-Gel column (8 × 22 cm) was prepared with 1100 ml of gel and successively washed with 300 ml of 1 mM Tris-HCl, 300-400 ml of 10 mM Tris-HCl, 300 ml of 0.5 mM Tris-HCl containing 10 mM Na2S2O3, and 400 ml of 10 mM Tris-HCl. After the crude extract was loaded on the column, it was washed with 500 ml of 10 mM Tris-HCl and the proteins were eluted with two Tris-HCl linear gradients (1 liter of 10 mM Tris-HCl and 1 liter of 0.25 mM Tris-HCl; 1 liter of 0.22 mM Tris-HCl and 1 liter of 0.4 mM Tris-HCl).

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The hydrogenase was collected in approximately 10-ml fractions between 2500 and 2800 ml.

First Hydroxylapitate Column.—A hydroxylapitate column (4.5 × 30.5 cm) was prepared with 485 ml of gel and washed successively with 300 ml of 1 M potassium phosphate buffer (KPB), 100 ml of 1 M Tris-HCl, 200 ml of 0.2 M Tris-HCl, 300 ml of 0.5 M Tris-HCl containing 10 mM Na2S2O4, and 300 ml of 0.2 M Tris-HCl. After the hydrogenase-containing fractions from the first column were applied, the column was washed with 50 ml of 10 mM KPB and the proteins were eluted with two phosphate linear gradients (1 liter of 10 mM KPB and 1 liter of 0.15 M KPB; 1 liter of 0.14 M KPB and 1 liter of 0.3 M KPB). The hydrogenase was eluted between 1600 and 2250 ml and concentrated to 90 ml in a Diaflow apparatus using a PM-30 membrane.

Second DEAE-Bio-Gel Column.—A DEAE-Bio-Gel column (6 × 21 cm) was prepared with 600 ml of gel and washed as previously described. The hydrogenase from the previous column was diluted with 150 ml of anaerobic water and applied to the column. After washing with 50 ml of 75 mM Tris-HCl, the column was developed with a Tris-HCl linear gradient (1 liter of 75 mM Tris-HCl and 1 liter of 0.25 M Tris-HCl). The hydrogenase eluted between 1310 and 1550 ml.

Second Hydroxylapitate Column.—A hydroxylapitate column (4.5 × 21 cm) was prepared with 334 ml of gel and washed as described for the first hydroxyapatite column. After the hydrogenase from the previous step was absorbed on the column, it was washed with 50 ml of 10 mM KPB and the column was developed with a linear phosphate gradient (1 liter of 0.15 M KPB) at a rate of 40 ml/h. The hydrogenase was eluted in two bands: hydrogenase I, between 1270 and 1390 ml and hydrogenase II, between 1710 and 1890 ml.

RESULTS AND DISCUSSION

The purification of the hydrogenases from *D. desulfuricans* is summarized in Table I. The overall recovery of hydrogenase activity was 13% and was divided about equally between two fractions containing hydrogenase activity, termed hydrogenase I and hydrogenase II. Hydrogenase II was found to have higher specific activity than hydrogenase I. Hydrogenase II was demonstrated to have a molecular weight of 75,500 by ultracentrifugation and was judged to be homogeneous by polyacrylamide disc electrophoresis. On the basis of sodium dodecyl sulfate-polyacrylamide electrophoresis, both hydrogenases were demonstrated to be composed of two polypeptide chains. Hydrogenase II contains 10.9 atoms of iron, 11.4 atoms of acid-labile sulfur and 0.6 atom of Ni per molecule. Hydrogenase I has a molecular weight of 77,600 by ultracentrifugation and appeared to be homogeneous by polyacrylamide disc electrophoresis. In addition to chromatographic behavior and specific activity, hydrogenase I differs from hydrogenase II in containing 7.8 atoms of iron, 6.8 atoms of acid-labile sulfur, and 0.6 atom of Ni per molecule. Since hydrogenase II exhibited the highest specific activity, it was subjected to detailed EPR and Mössbauer studies discussed below.

### Table I

| Purification of hydrogenases from *D. desulfuricans* |
|-----------------------------------------------|
| **Fraction** | **Volume (ml)** | **Protein (mg)** | **Total units** | **Specific activity (µmol H₂/min/mg)** | **Recovery (%)** |
| Crude extract | 1.05 × 10⁴ | 22.7 × 10³ | 48.2 | 3.2 | | |
| First DEAE-Bio-Gel column | 675 | 6.44 × 10³ | 36.6 | 5.69 | 76 |
| First Hydroxylapitate column | 575 | 590 | 15.42 | 26.1 | 32 |
| Second DEAE-Bio-Gel column | 240 | 158 | 9.76 | 61.6 | 20 |
| Second Hydroxylapitate column | | | | | |
| Hydrogenase I | 0.68 | 28 | 2.72 | 97.0 | 5.6 |
| Hydrogenase II | 0.29 | 24 | 3.68 | 152 | 7.6 |

*61Ni- and 57Fe-enriched Hydrogenase from *D. desulfuricans**

The EPR spectra of purified hydrogenase II as isolated from cells of *D. desulfuricans* grown on both unenriched, and *61Ni- and 57Fe-enriched media are compared at two temperatures, 11 and 90 K. Fig. 1A shows the spectra recorded at 90 K. The signals at 2.32 and 2.16 show significant broadening for the *61Ni-enriched hydrogenase when compared to the naturally occurring purified hydrogenase. In the g = 2.01 region, hyperfine structure is observed for the *61Ni-enriched protein. Lancaster (11) reported four distinct hyperfine lines in this region in the *M. bryantii* *61Ni-enriched system due to the nuclear spin (I = ⁷/₂) of *61Ni. It is interesting to note that the separation between the two outermost lines for the *61Ni-enriched hydrogenase is 79 G while that measured from Lancaster’s data is 80 G. Thus, the hyperfine patterns for *61Ni show both broadening and partial resolution of hyperfine lines indicating unequivocally that the rhombic signal observed in the oxidized state of hydrogenase is due to nickel.

Since the inner two hyperfine lines are not well resolved, and the enzyme is also enriched in *¹⁷Fe, the observed hyperfine pattern could be due to the nuclear spin (I = ⁵/₂) of *¹⁷Fe. However, a hyperfine splitting of 80 G observed in an EPR spectrum for an electronic spin ½ system corresponds to an internal field of 800 kG at the *¹⁷Fe nucleus. The following Mössbauer data (Table II) completely rule out this possibility.

Fig. 1B shows the EPR spectrum of purified hydrogenase recorded at 11 K in the g = 2.02 region. Hyperfine broadening due to the *¹⁷Fe nuclear spin is very definitely observed in the *¹⁷Fe-enriched hydrogenase. The half-width signal of the *¹⁷Fe-hydrogenase signal is 21 G while that of the *¹⁷Fe-enriched hydrogenase signal is 32 G, indicating a broadening of 11 G. There also appears to be hyperfine structure in the *¹⁷Fe-enriched hydrogenase spectrum with an apparent splitting constant of approximately 13 G. These values of splitting constants are within the range of those found in iron-sulfur clusters, and agree very well with the magnetic hyperfine coupling constants obtained for a paramagnetic component observed in the *¹⁷Fe Mössbauer spectra (Table II).

Upon reduction by hydrogenase, the isotropic g = 2.02 signal and the rhombic Ni signal disappear and are replaced by an intense "g = 1.94 type reduced [Fe₄S₄]" signal and additional weak signals presumably due to nickel. The new
The asymmetry parameter.

Gain (natural abundant Fe hydrogenase)

The limited resolution, the A-tensor is assumed to be isotropic for site metal at room temperature) are values obtained at [4Fe-4S] cluster (20, 21).

The quadrupole splittings \( \Delta E_Q \) and isomer shifts \( \delta \) (relative to iron metal at room temperature) are values obtained at 85 K. \( g \) is the nuclear magnetic moment. With the limited resolution, the A-tensor is assumed to be isotropic for site 2 and is undetermined for site 3. The values in parentheses are equivalent gauss at the electron. These values are consistent with the hyperfine separations observed in the isotropic \( g = 2.02 \) signal.

| Iron site | \( A_1/g_BA_1 \) (kG) | \( A_2/g_BA_2 \) (kG) | \( A_3/g_BA_3 \) (kG) | \( \Delta E_Q \) (mm/s) | \( \eta \) | \( \delta \) (mm/s) |
|-----------|------------------|------------------|------------------|------------------|--------|------------------|
| 1         | 300              | 325              | 325              | 0.63             | 1      | 0.36             |
| (15 G)    | (16 G)           | (16 G)           |                  |                  |        |                  |
| 2         | 130              | 130              | 130              | 0.63             | 0      | 0.36             |
| (6 G)     | (6 G)            | (6 G)            |                  |                  |        |                  |
| 3         |                  |                  |                  | 0.63             |        | 0.36             |

The nickel signal has maximum \( g \) value at 2.28 and its intensity is about one-third of the \( g = 2.32 \) resonance. These observations will be the subject of a future communication.

Fig. 2 shows Mössbauer spectra of Fe-enriched hydrogenase II from D. desulfuricans. The data are recorded at 4.2 K with a magnetic field of 500 G applied parallel (Fig. 2A) and perpendicular (Fig. 2B) to the \( \gamma \)-radiation. The spectra are recorded at 4.2 K with a magnetic field of 500 G applied parallel (A) and perpendicular (B) to the \( \gamma \)-radiation. A difference spectrum of spectra A and B is shown in C (see text). The brackets indicate the two pairs of \( \Delta m = 0 \) nuclear transitions. The solid lines are theoretical spectra for the two discernible iron sites of the \( g = 2.02 \) paramagnetic center. The parameters used for the theoretical computations are listed in Table II.
The activation in the transformation of 3Fe clusters into [4Fe-4S] clusters (33).

The EPR studies showed that at temperatures higher than 77 K, the g = 2.02 signal disappeared, indicating fast electronic relaxation rate. Consistent with this EPR observation, the Mössbauer magnetic component collapses into a sharp quadrupole doublet at 85 K. The measured parameters (ΔE_Q = 0.63 mm/s and δ = 0.36 mm/s) are typical for high spin ferric ion (S = 5/2). However, two S = 5/2 ions cannot be coupled to form a total spin of ½. A third half-integral spin is required. Also the absorption intensity of the collapsed quadrupole doublet was found to be 25-30% of the total absorption, which is about ½ of that of the two discernible magnetic iron sites. We therefore conclude that the g = 2.02 center must contain a third iron site which at low temperature spectrum is masked by the intense central doublet.

Three-iron clusters have recently been found in various proteins (23-25). The physical properties of the g = 2.02 center in hydrogenase are very similar to those of a 3Fe cluster found in Azotobacter vinelandii ferredoxin (23). However, all the oxidized 3Fe clusters reported so far have an isomer shift less than 0.3 mm/s, a typical value for high spin ferric ion in a tetrahedral sulfur environment. In D. desulfuricans hydrogenase a relatively larger isomer shift, 0.36 mm/s, was found, suggesting that the iron coordination of this trinuclear iron cluster may contain nitrogenous or oxygentic ligands.

The hydrogenases isolated from D. gigas (2, 26, 27), D. furicans, and the hydrogenase of D. vulgaris (28, 29) exhibit significant differences in their molecular organization and specific activity. However, evidence collected from chemical analyses, EPR, and Mössbauer studies indicate that all three enzymes contain some common prosthetic groups. All three hydrogenases were reported to contain approximately 11-12 non-heme iron and comparable amounts of acid-labile sulfurs per molecule. Approximately 1 atom of nickel was found in hydrogenase from D. gigas (2) and from D. furicans but there has been no report of Ni in hydrogenase from D. vulgaris. The EPR and Mössbauer spectra of isolated hydrogenases from D. gigas and from D. desulfuricans are practically the same, suggesting similar organization for the iron and sulfur atoms in the enzymes (i.e. two [4Fe-4S] clusters and one 3Fe cluster per molecule). As isolated, the hydrogenase from D. vulgaris exhibits a g = 2.02 EPR signal which is lost upon reduction and replaced by a low intensity g = 1.94 type signal (3). These signals are consistent, respectively, with oxidized 3Fe cluster and reduced ferredoxin type [4Fe-4S] cluster.

Recently, it has been shown that [4Fe-4S] clusters can be converted into 3Fe clusters by ferricyanide oxidation (30-32). The activation of beef heart aconitase was shown to involve the transformation of 3Fe clusters into [4Fe-4S] clusters (33).

All symbols in Equation 1 have their conventional meanings (22). The solid lines in Fig. 2 are theoretical simulations. The measured parameters for calculations are listed in Table II. By comparing the absorption intensities of the peaks at -3 and -2 mm/s with those of the simulated spectra, the two iron sites are estimated to contribute 16-20% of the total iron content.