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THE PRESENCE OF REDOX-SENSITIVE NICKEL IN THE PERIPLASMIC HYDROGENASE FROM DESULFOVIBRIO GIGAS

J. LeGall[a], P. O. Ljungdahl[a], I. Moura[b], H. D. Peck, Jr.[a], A. V. Xavier[b], J. J. G. Moura[b], M. Teixera[b], B. H. Huynh[c] and D. V. DerVartanian[a].

Department of Biochemistry, University of Georgia, Athens, GA, 30602[a], Centro de Quimica Estrutural das Universidades de Lisboa I. St. 1000 Lisbon, Portugal[b] and Department of Physics, Emory University, Atlanta, GA, 30322.[c]

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SUMMARY: A new and improved method for the purification of the periplasmic hydrogenase from Desulfovibrio gigas is described. This preparation of hydrogenase was found to contain 0.64 g atom of nickel per mole of protein. In the oxidized state, the hydrogenase exhibited an isotropic signal at g = 2.02 and a characteristic Ni(III) signal with g-values at 2.31, 2.20 and ∿2.0. The EPR spectrum of the reduced enzyme consisted of multiple species. One set of g-values are determined as 2.17, 2.08 and 2.04. The other minor species exhibited a resonance at g = 2.28. On partial reoxidation of the hydrogenase, the initial Ni(III) signals reappeared along with additional signals attributed to multiple Ni(III) species. It is proposed that Ni is an important functional unit in this hydrogenase.

INTRODUCTION:

The periplasmic hydrogenase of the sulfate reducing bacterium, Desulfovibrio gigas (1) has been purified to apparent homogeneity (2) and shown to contain 12 non-heme irons and sulfides arranged in 3[Fe_4S_4] clusters per molecular weight of 89,500 daltons. The enzyme differs from the purified periplasmic hydrogenase of D. vulgaris (Mr = 50,000) (3,4) in exhibiting a lower specific activity (∿100 vs 3800), having two subunits (Mr = 62,000 and 26,000) and a higher molecular weight. Their EPR spectra exhibit significant similarities which are common to several other hydrogenases (5). In the oxidized state (as isolated) both enzymes exhibit a g = 2.02 signal which is lost upon reduction with dithionite with mid-point potentials up to +25 mV. This type of signal is consistent with either a high potential [Fe_4S_4] cluster (6) or an [Fe_3S_3] cluster (7,8). The reduced hydrogenases show either a low intensity or no g = 1.94 signal (9).

In this communication, we report the presence of Ni in the hydrogenase of D. gigas purified by a new procedure and the first demonstration of redox sensitive nickel in a pure protein.

METHODS

Preparation of hydrogenase. We report here a new simplified procedure for the purification of the periplasmic hydrogenase of D. gigas (Table I): For all purifica-

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Table I. Purification of Hydrogenase from D. gigas

	Volume	Specific* Activity	Total Units	Recovery
Step 1: crude extract	64 ml	1.49	16,700	100
Step 2: first DEAE column	253 ml	2.60	12,010	72
Step 3: Hydroxylapatite column	378 ml	11.00	12,000	72
Step 4: second DEAE column	2.7	90.3	9,058	54

*Expressed in μmoles H_2 evolved mg^{-1} min^{-1}.

tion steps, the temperature was maintained at 5°C and the pH of the buffers was at 7.6 (measured at 20°C for Tris-HCl). Precautions were taken against oxygen by flushing buffers with purified argon and by running 100 ml of buffer containing 1 mM sodium dithionite through each column and then washing them with 100 ml of O_2-free buffer.

Step 1: Crude Extract: In a typical experiment, 280 g wet weight of D. gigas cells, stored several weeks at -80°C, were thawed under Ar and centrifuged for 20 min at 30,000 x g giving 50 ml. of liquid. The cells were washed twice with 50 ml of 0.05 M Tris-HCl. The three extracts were combined giving a total volume of approximately 200 ml. This extract was concentrated in a Diaflow apparatus with a YM-30 membrane to 50 ml and dialyzed against 500 ml of Tris-HCl buffer at 0.005 M, giving 64 ml of extract.

Step 2: First DEAE-Biogel Column: A DEAE-Biogel column was prepared with 500 ml of gel; after deposition of the extract, the column was washed with 100 ml of Tris-HCl (0.01 M/750 ml Tris-HCl (0.25 M). Hydrogenase was collected in a volume of 253 ml.

Step 3: Hydroxylapatite Column: A Biogel-HTP hydroxylapatite column (2.5 cm x 20 cm) was equilibrated with 0.15 M Tris-HCl. The proteins were eluted with a potassium phosphate buffer gradient (750 ml of 0.01M and 750 ml of 0.25M). The hydrogenase activity was recovered in a volume of 378 ml and dialyzed against 1 liter of 5 mM Tris-HCl buffer.

Step 4: Second DEAE-Biogel Column: The protein was next absorbed on a column identical to the one utilized in step 2. The proteins were eluted with a linear Tris-HCl gradient (750 ml of 0.01M and 750 ml of 0.2M). The fractions containing the highest specific activity (90.3 units mg^{-1} min^{-1}) were collected in a volume of 52 ml and were concentrated to 2.7 ml using a Diaflo apparatus as before. The yield was 104 mg of hydrogenase with an absorbance ratio 400 mm, 280 nm of 0.28 and a single protein band in 7.5% acrylamide gels (10).

Nickel was determined by plasma emission spectroscopy using the Jarrell-Ash Model 750 atomcomp.

EPR spectroscopy was conducted with a Varian E-109 spectrometer interfaced with a Hewlett-Packard HP-85 microcomputer. Measurements in the liquid-helium temperature range were performed with an Air Products APD-E automatic temperature controller. Other EPR experimental conditions are found in Figure Legends.

Hydrogenase activity was routinely measured by the H_2 evolution assay from reduced methyl viologen (11). The final concentrations of reagents in a 3 ml assay

volume were as follows: 1 mM methyl viologen; 15 mM $Na_2S_2O_4$; 1 mg/ml BSA to stabilize the protein and 50 mM TRIS at pH 7.6. The assay was carried out at 32°C under an Argon atmosphere with vigorous shaking.

The assay mixture was prepared under anaerobic conditions in 15 ml Hypovials (Pierce Chemical Company, Rockford, IL, 61105 U.S.A.) which were closed with butyl rubber serum stoppers (Bellco Glass, Inc., Vineland, NJ, 08360 U.S.A.) with aluminum seals (Pierce Chemical Company). These assay vials were prepared in large quantities and stored at -20°C until needed.

The assay was initiated by injecting 10 µl hydrogenase preparations through the rubber serum stoppers. At 5 min. and at 10 min. 250 µl of the gas phase was injected into a Varian 4600 gas chromatograph and H_2 was quantified by the Peak Height Method using a Varian CDS 401 Data Station.

RESULTS AND DISCUSSION:

The EPR spectrum of isolated hydrogenase (Figure 1A) consists of an isotropic EPR signal at g = 2.02 and a rhombic signal with g-values at 2.31, 2.20 and ∿2.0. A minor species (as noted in Figure 1A) can be seen at g = 2.32 and 2.13 but the intensity of this latter signal varies with preparations. It is interesting to note that the rhombic signal saturates at 50 µW when measured at 11 K. An isotropic g = 2.02 signal is consistent with that of an oxidized [Fe_3S_3] cluster (7,8) or a superoxidized [Fe_4S_4] cluster (6); however, preliminary Mössbauer studies show that these clusters are absent in the isolated enzyme. An alternate possibility is that the isotropic signal originates from a yet uncharacterized iron-sulfur cluster. The rhombic EPR signal found in hydrogenase has g-values, line-shape and saturation properties which are similar to those reported for membranes of Methanobacterium bryantii. By comparison with model nickel complexes, Lancaster (13) tentatively attributed this EPR signal to Ni(III). Using isotropic substitution of ^{61}Ni(I=3/2) into oxidized membranes, Lancaster (14) unequivocally established that the rhombic EPR signal was due to nickel because of induced nuclear hyperfine structure. On the basis of his observation, our detection of a nearly identical EPR spectrum and the chemical determination of nickel in purified hydrogenase, we attribute the signal with g-values at 2.31 and 2.20 to a Ni(III) complex.

Plasma emission spectroscopy has detected 0.64 g atom of nickel per 89,500 g protein. Quantitation by double-integration of the nickel(III) EPR signal showed a recovery of 0.33 g atom of nickel per mol protein which corresponds to a recovery by EPR quantitation of 52% of the chemically determined nickel content of purified hydrogenase.

When hydrogenase was reduced under anaerobic conditions with hydrogen (Figure 1C) both the rhombic signal attributed to the Ni(III) complex and the isotropic g = 2.02 signal disappeared and were replaced by a complex EPR spectrum. The EPR spectrum consists of multiple species with one set of g-values of 2.17, 2.08 and 2.04 while the other species had a major g-value of 2.28. The latter species was not always present but varied with the hydrogenase preparation. Spin quantitation of the g = 2.17, 2.08 and 2.04 signal showed 0.25 spin per 89,500 g protein. Variation of signal intensity with microwave power at 11 K indicated that the EPR signal did not

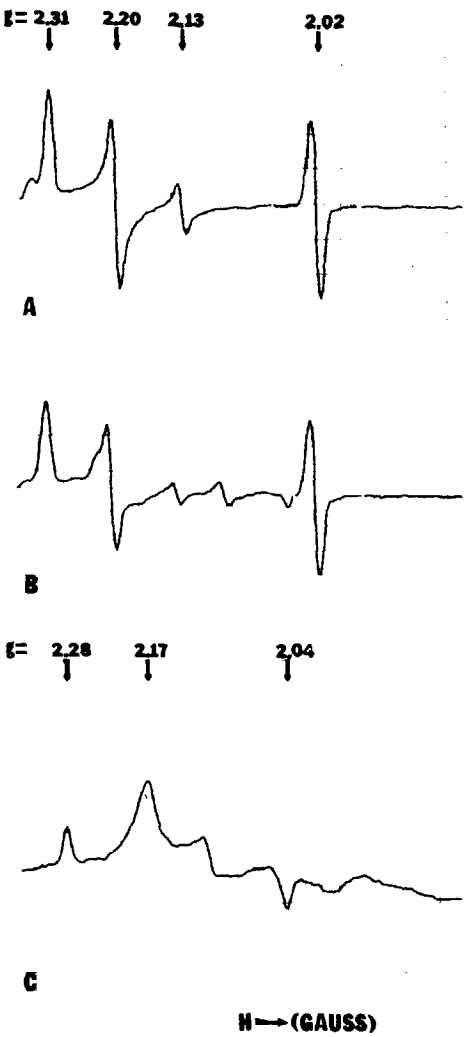

Figure 1. EPR Spectra of Hydrogenase Purified from D. Gigas

A. Purified Hydrogenase (180 μM in protein in 150 mM potassium phosphate buffer, pH 7.6). EPR conditions: microwave power, 20 μW; microwave frequency, 9.157 GHz; modulation amplitude, 10 G; temperature, 11 K; scanning rate, 1000 G per min; time constant, 0.1 sec; Gain = 1.6×10^4. The EPR signal at g = 2.02 was recorded under the same EPR conditions except that the Gain = 3.2×10^2.

B. Purified Hydrogenase (400 μM in protein in the above buffer), previously fully reduced with hydrogen was then subjected to vacuum-argon cycling to remove hydrogen and allowed to stand at 25°C for 8 hours under argon. EPR conditions as in (A) except that the microwave power was 50 μW; microwave frequency, 9.161 GHz; and Gain = 1.0×10^4. The EPR signal at g = 2.02 was measured under the same EPR conditions except that the Gain = 1.0×10^3.

C. Purified Hydrogenase at the same protein concentration as in (A) was reduced under hydrogen for 8 hours. EPR conditions as in (A) except the microwave frequency was 9.163 GHz, the microwave power was 30 μW and the Gain = 1.25×10^4.

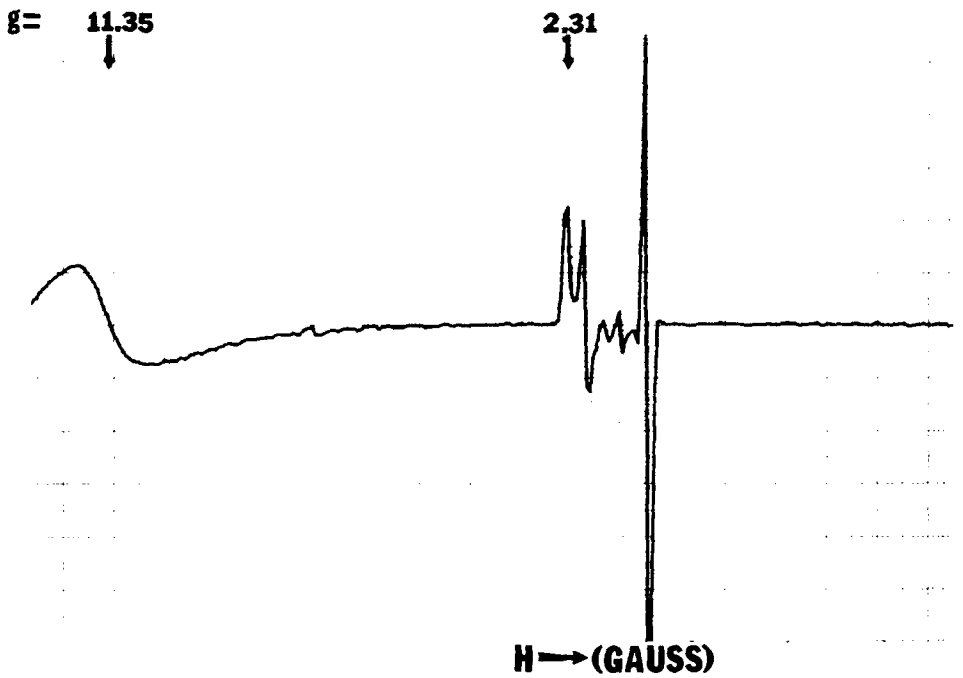

Figure 2. EPR Spectrum of Hydrogenase at the Intermediate Redox State of Figure 1 B. EPR conditions as in Fig. 1B except the scan range was five-fold expanded; microwave power, 50 μW; microwave frequency, 9.161 GHz and Gain = 1.6 X 10^4.

saturate at 50 mW which is in contrast to the initial Ni(III) signal of isolated hydrogenase. Based on the g-values we tentatively assign this complex EPR signal to transient Ni(III) species. It is intriguing that hydrogenase reduced by dithionite plus trace amounts of methyl viologen under argon neither exhibited this complex EPR signal nor evolved H_2. We therefore tentatively suggest that this complex signal is due to an altered Ni(III) species and that the observed EPR changes represent interaction of nickel with H_2.

Figure 1B shows the EPR spectra when reduced hydrogenase (with hydrogen) was allowed to reoxidize (by multiple cycling using vacuum then argon and finally left in an argon atmosphere) for eight hours. The initial Ni(III) EPR signals reappeared with additional signals which can be attributed to multiple Ni(III) species with g-values of 2.09 and 2.02. Simultaneously the isotropic EPR signal at g = 2.02 has also reappeared but has not reached the intensity of the signal found in the isolated hydrogenase. Figure 2 shows an expanded EPR spectrum of hydrogenase at the above intermediate redox level. There appears a novel and intense EPR signal at g = 11.35. At 11 K, the signal at g = 11.35 is not saturated at 20 mW. Because of the very high g-value, it is likely that the signal may arise from a spin-coupled metal center or from strong-coupling between centers.

Nickel has only been recently recognized as an important trace element in biological systems (15). The element is required for the growth of several physiological

types of microorganisms (16,17) and for the biosynthesis of hydrogenase (18), CO dehydrogenase (19) and factor F_{430} (20). Nickel has now been shown to be an integral component of CO dehydrogenase (21), factor F_{430} (22,23,24) and the hydrogenase of M. thermoautotrophicum (25). This partially purified hydrogenase (Mr = 60,000) was demonstrated to contain approximately one g atom of nickel per mol of hydrogenase using ^{61}Ni; however, no information was presented on the role of nickel in the redox mechanism of hydrogenase. A reduction of the nickel in F_{430}, a component of methyl Co-M reductase (26), by dithionite has not been detected (27,28). Similarly, a redox cycling of the nickel in CO dehydrogenase has not been reported.

Our chemical analysis for nickel shows that the pure hydrogenase from D. gigas contains 0.64 g atom per mol of hydrogenase. The EPR recovery of the nickel EPR signal indicates that a substantial amount of the nickel in the isolated enzyme is in the Ni(III) state. Furthermore, EPR studies show that nickel is sensitive to the redox state of the protein. It is very likely then that nickel is an important catalytic component of hydrogenase, and may represent the binding site for the substrate, H_2.

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