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Electron Paramagnetic Resonance (EPR) Studies on Hydrogenase-1 (HYD1) Purified from a Mutant Strain (AP6) of *Escherichia coli* Enhanced in HYD1

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Hydrogenase-1 (HYD1), overexpressed by twofold, has been purified to homogeneity and to a high specific activity from a mutant strain (AP6) of *Escherichia coli* which lacks hydrogenase-2. Plasma emission spectroscopy indicated that 0.93 atom of nickel and 11.4 iron atoms were present in HYD1. EPR studies on the as isolated HYD1 detected a complex 3Fe-4S signal and a Ni(III) species. Reduction with hydrogen gas caused disappearance of both the 3Fe–4S cluster and initial Ni(III) signals. At the same time the EPR signature (small g=2.19 signal) of the activated hydrogenase appeared. The detection of a 4Fe–4S cluster signal was noted. Reduction of HYD1 with sodium dithionite caused all nickel signals to disappear. The 4Fe–4S complex intensity was slightly increased. The EPR responses in the three oxidation-reduction states are consistent with other known (NiFe)-hydrogenases.

Under anaerobic conditions, *Escherichia coli* produces three different nickel-containing hydrogenases (*cf.* refs. 1,2). Hydrogenase 3 (HYD3) is responsible for formate-dependent dihydrogen evolution (3). HYD2 is involved in hydrogen uptake and has been purified and found to be a heterodimeric enzyme with a 58-kDa large subunit and a 30-kDa small subunit (4). HYD1 is believed to be involved in hydrogen cycling during fermentative growth. HYD1 has been purified and found to contain a 60-kDa large subunit and a 30-kDa small subunit (5,6). The operon encoding the two structural subunits of HYD1 contains a total of six genes and has been mapped at 22 min on the *E. coli* chromosome (7,8). Operons encoding HYD2 and HYD3 have also been identified. Besides the operons coding for the structural components of the three hydrogenases, a fourth operon (*hyp*) is required for enzymatic activity for all three hydrogenases. At least one of the genes in *hypB* is involved in nickel metabolism, most probably by nickel insertion into apoenzyme (9).

We have previously mutagenized the gene for the large subunit of HYD1 and analyzed crude extracts and membranes of mutants for the presence of active HYD1. These site-directed mutagenesis studies established Cys 76, Cys 576 and Cys 579 as possible ligands for the nickel moiety (1). These assignments were essentially confirmed by X-ray crystallography on a similar (Ni-Fe) hydrogenase from *Desulfovibrio gigas* (10).

In the present study HYD1 has been purified to homogeneity from a high yield HYD1 mutant strain (AP6). A higher yield of HYD1 facilitates EPR studies because of the high concentrations of enzyme needed to detect paramagnetic species. These studies have been performed to establish that HYD1 has spectroscopic properties similar to other (Ni-Fe) hydrogenases. To our knowledge these EPR studies are the first to have been conducted on any hydrogenase from *E. coli.*

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METHODS

Construction of strain with a higher yield of HYD1. One of the problems encountered during purification of HYD1 is that HYD2 activity contaminates HYD1 fractions. To reduce the number of steps for purification of HYD1 and increase yields, an *E. coli* strain was constructed which contained only HYD1 activity. Studies on mutagenesis of the *hyb* operon showed that the *hyb* deletion mutants showed consistently significantly lower levels of HYD1 activity, indicating that the protein encoded by one or more genes of the *hyb* operon were required for normal HYD1 activity. HYD1 activity could be restored to normal levels when the *hyb* deletion mutant was complemented with a plasmid containing an in-frame deletion of the HYD2 large subunit. This indicated that active HYD2 *per se* was not required for restoration of HYD1 activity.

A hydrogenase-negative *E. coli* strain was cotransformed with pBR 322 carrying the *hya* operon inserted into the Scal site of the β lactamase gene (tet +) and pACY184 carrying a *hybb* in-frame deletion (2). This strain was analyzed and found to exhibit approximately two-fold higher HYD1 levels than the parent TG1 strain and hence was used for further studies. This strain has been designated as AP6.

Purification and characterization of HYD1. *E. coli* AP6 cells were grown anaerobically overnight in a 100 L or 400 L fermenter on 0.4% glucose at 37 C. Membranes were prepared from cells subjected to the Gaulin Press and later treatment with 0.001% DNase. Triton X-100 (final concentration of 2%) was added to the membranes (in 50 mM Tris buffer, pH 7.2). Suspension was stirred overnight in the cold room. The membranes were extracted a second time with Triton X-100 under the same conditions as described for the first extraction. The combined brown membrane extracts were dialyzed overnight in the cold room and applied consecutively to two Poros IIQP anion exchange columns followed by preparative native polyacrylamide gel electrophoresis and hydroxylapatite chromatography. From 320 g (wet paste) of *E. coli* AP6 cells were produced 625 ml of brown membrane extract (containing HYD1 enzymatic activity), 2601 mg protein (determined by the Bradford procedure, ref. 11)and a total activity of 788 units per minute which resulted in a specific activity of 0.3 unit per mg protein per minute. Hydrogenase activity and unit definition was determined by the hydrogen-benzyl biologen oxidoreductase method as described by Sawyer and Boxer (5).

After hydroxylapatite chromatography, the total amount of pure HYD1 recovered was 6.7 mg with a specific activity of 26.5 units per mg protein per minute. Plasma emission spectroscopy (Jarrell-Ash 965 Atomcomp Plasma Emission Spectrograph) determined 0.93 atom of nickel and 11.4 iron atoms per mole of HYD1. These metal values are in

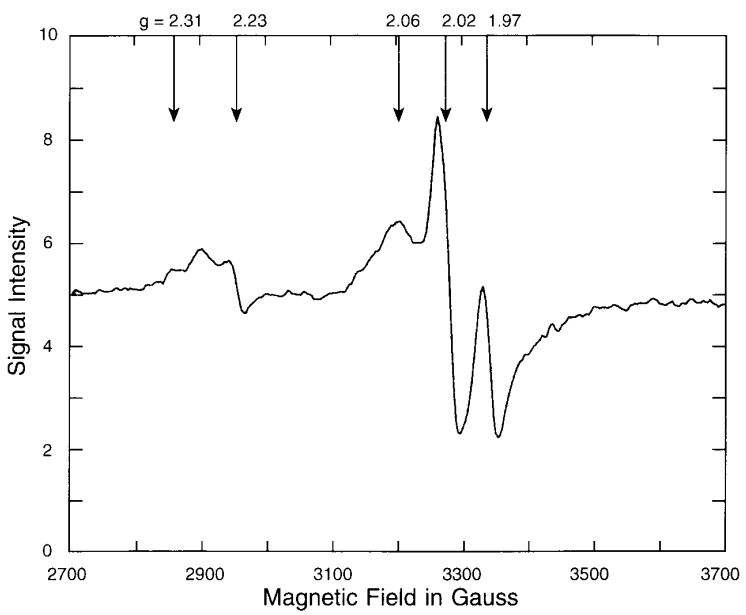

FIG. 1. EPR spectrum (Measured with Varian E-109 spectrometer and Air Products Low Temperature system) of as isolated HYD1 (10 μM in protein and in 50 mmM Tris buffer, pH 7.2). EPR conditions: microwave power, 1 mW; scan rate, 250 gauss per min; temperature, 19 K; time constant, 0.3 sec; microwave frequency, 9.24 ghz; gain, 50,000.

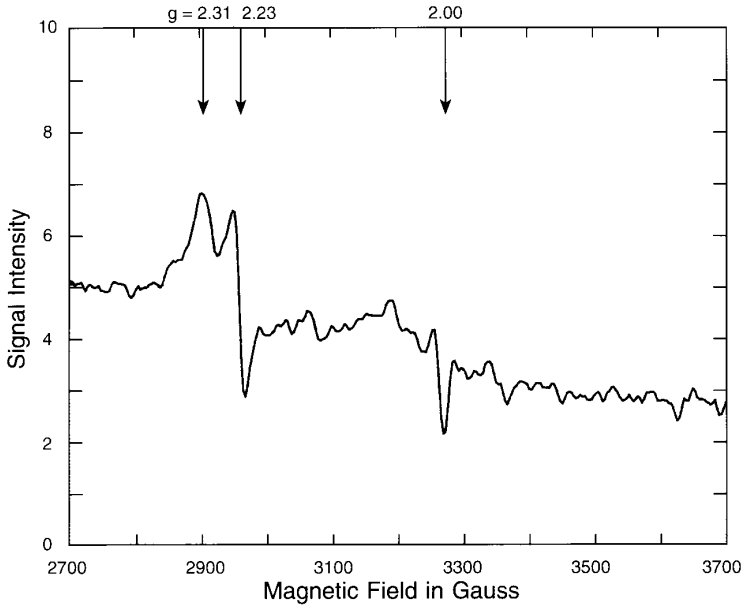

FIG. 2. EPR spectrum of an isolated HYD1; same protein and buffer concentrations as in Fig. 1. EPR conditions: same conditions as in Fig. 1 except microwave power, 10 mW; temperature, 50 K; microwave frequency, 9.26 ghz; gain, 50,000.

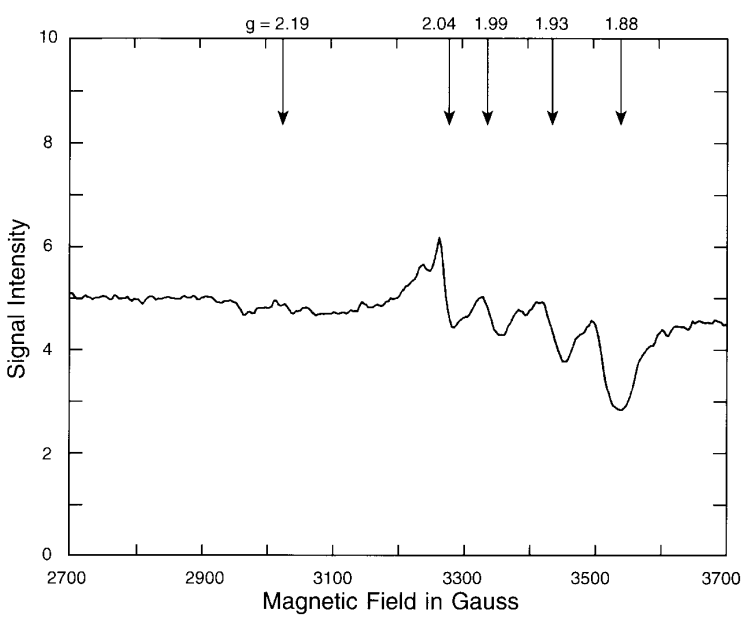

FIG. 3. EPR spectrum of HYD1 (12 μM in protein; same buffer concentration as in Fig. 1) reacted under anaerobic conditions with hydrogen gas for 2 hours. EPR conditions: same conditions as in Fig. 1 except microwave power, 0.5 mW; temperature 19 K; gain, 63,000.

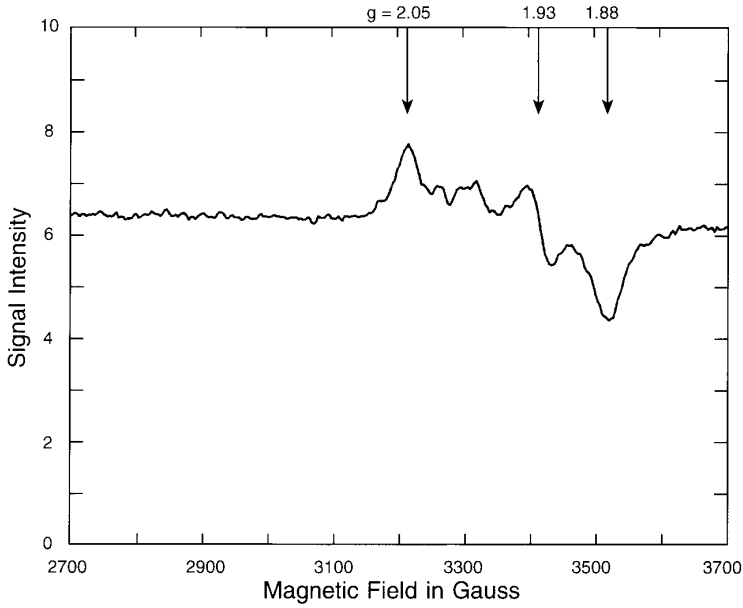

FIG. 4. EPR spectrum of HYD1 (protein and buffer concentrations as in Fig. 3) reduced under anaerobic conditions with slight excess of solid sodium dithionite. EPR conditions are the same as in Fig. 3.

general agreement with those reported by Sawyer and Boxer (5). However the specific activity and yield of HYD1 was 2.5 and 2.0 fold higher, respectively, than that reported by Sawyer and Boxer (5).

RESULTS AND DISCUSSION

EPR Studies on E. coli Hydrogenase (HYD1) Oxidized State

EPR studies on as isolated HYD1 have been carried out at liquid helium temperatures and reveal complex highly temperature sensitive signals with g-values at 2.06, 2.02 and 1.97 (see Fig. 1). In general hydrogenases with a 3Fe-4S cluster exhibit an isotropic signal at g=2.02. The EPR behavior, g-values and oxidation state of HYD1 suggest that the complex signal at g=2.02 arises from a 3Fe-4S cluster. The associated additional signals at g=2.06 and 1.97 very likely represent spin coupling between the iron atoms in the 3Fe-4S cluster and the nickel species.

A second rhombic resonance is also readily discernible with g-values at 2.31 and 2.23 and is attributable to a Ni(III) species. This latter signal has been seen in a number of (Ni-Fe) hydrogenases such as hydrogenase from *Desulfovibrio gigas* (11).

The complex signal centered at g=2.02 disappears when measured at 50K and now the entire rhombic Ni(III) species can be observed with an additional g-value at 2.00 (see Fig. 2). Spin quantitation by double integration accounts for approximately one spin for the complex g=2.02 signal and one spin for the Ni(III) species.

Hydrogen-Reduced State

When HYD1 was reduced under anaerobic conditions with hydrogen gas for 2 hours, the EPR signals of the complex 3Fe-4S cluster decreased significantly and were replaced by new signals at g=2.04, 1.93 and 1.88 indicating the appearance of a low-potential 4Fe-4S cluster (see Fig. 3). The initial Ni(III) signal has disappeared and was replaced by the EPR ''signature'' of the activated hydrogenase system represented by a small g=2.19 signal (1).

Sodium Dithionite Reduced State

When HYD1 was reduced under anaerobic conditions with a slight excess of sodium dithionite, all signals attributable to the 3Fe-4S cluster (centered at g=2.02) and to the Ni(III) species disappeared and were replaced by a reduced 4Fe-4S cluster signal with g-values at 2.05, 1.99 and 1.93 (see Fig. 4). The spin quantitation of this latter cluster also accounted for approximately one spin per mole HYD1. The absence in detection of a second 4Fe-4S cluster signal may be attributed to spin interaction between the Fe-S clusters or alternatively the other Fe-S cluster may have a more negative oxidation-reduction potential when prevents reduction by sodium dithionite. The EPR resonances for both the hydrogen-reduced or sodium dithionite-reduced states are nearly superimposable on the EPR spectrum reported for the hydrogen-reduced state of hydrogenase from *Azotobacter vinelandii* (12). In conclusion, the mutant strain (AP6) has been devised to increase the yield of HYD1 necessary to perform biophysical measurements. Overexpression of highly active HYD1 has been achieved and the correct number of nickel and iron atoms have been inserted into HYD1. The EPR resonances observed in the three oxidation-reduction states of HYD1 are consistent with EPR resonances observed in typical (Ni-Fe) hydrogenases (1,13).

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