Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3728–3732, June 1984
Biophysics

Desulfovibrio vulgaris hydrogenase: A nonheme iron enzyme lacking nickel that exhibits anomalous EPR and Mössbauer spectra

(iron-sulfur protein)

BOI HANH HUYNH*, MELVIN H. CZECHOWSKI†, HANS-JÖRG KRÜGER†, DANIEL V. DERVARTANIAN†, HARRY D. PECK, JR.†, AND JEAN LEGALL†

*Department of Physics, Emory University, Atlanta, GA 30322; and †Department of Biochemistry, University of Georgia, Athens, GA 30602

Communicated by Martin D. Kamen, March 12, 1984

ABSTRACT **A purification procedure for the periplasmic hydrogenase from *Desulfovibrio vulgaris* (Hildenborough, National Collection of Industrial Bacteria 8303) is reported. The purified hydrogenase has a specific activity of 4800 units per mg of protein. Plasma emission studies reveal that this highly active hydrogenase is free of nickel and contains 11 (±1) nonheme iron atoms per molecule. A combined EPR and Mössbauer study indicates that the majority of the iron atoms are bound in the form of iron–sulfur clusters. Two ferredoxin-type [4Fe–4S] clusters have been identified that exhibit normal EPR and Mössbauer parameters; however, no trace of 3Fe cluster is detected by the Mössbauer measurement. In the presence of oxidants, cytochrome c_3, and CO, anomalous EPR and Mössbauer spectra indicative of atypical nonheme iron centers are observed.**

The periplasmic hydrogenase from *Desulfovibrio vulgaris* is composed of a single polypeptide chain, M_r 50,000, contains 12 nonheme irons, and exhibits a high specific activity in the hydrogen evolution assay from reduced methyl viologen (1). In these respects, it appears to be more comparable to the hydrogenases of *Clostridium pasteurianum* (2) and *Megasphaera elsdenii* (3) than the nickel-containing periplasmic hydrogenase from *Desulfovibrio gigas* (4, 5) and *Desulfovibrio desulfuricans* (6), which have two subunits and exhibit a low specific activity in the evolution assay (6, 7). It has recently been reported that the hydrogenase of *D. vulgaris* contains variable amounts of diamagnetic nickel (0.3–0.8 atoms per molecule of enzyme) and that the 12 nonheme irons are arranged in three [4Fe–4S] clusters of the bacterial ferredoxin-type, based on EPR and core extrusion studies (8).

In this communication, we report the preparation of hydrogenase from *D. vulgaris* free of nickel and anomalous EPR and Mössbauer spectra that are indicative of two novel states of nonheme iron centers.

MATERIALS AND METHODS

Growth of Organisms and Preparation of Crude Extract. ^{57}Fe-labeled *D. vulgaris* (Hildenborough, NCIB 8303) was grown for 38 hr in lactate/sulfate medium containing the following components per liter: sodium lactate (60%), 12.5 ml; NH_4Cl, 2 g; $MgSO_4 \cdot 7H_2O$, 2 g; K_2HPO_4, 0.5 g; Na_2SO_4, 4 g; $CaCl_2 \cdot 2H_2O$, 0.035 g; $Na_2S \cdot 9H_2O$, 0.25 g; ^{57}Fe (enrichment, 95%), 1 mg; EDTA, 2 mg; cysteine hydrochloride, 0.125 g; mineral solution (9), 1 ml. The pH of the medium was 7.2 after autoclaving. Cells (407 g) from 200 liters were harvested and then suspended in 400 ml of 10 mM Tris·HCl (pH 7.6) and frozen at −80°C for 2 days. The cells were then slowly defrosted for about 20 hr and were centrifuged at 19,000 × *g* for 45 min. The supernatant was called crude extract.

Purification of Hydrogenase. All purification procedures were carried out in air, at 4°C, and the pH of the buffers was 7.6 (measured at 5°C). A summary of the results of a typical purification is presented in Table 1.

First DEAE-Bio-Gel column. The crude extract was placed on a DEAE Bio-Gel column (6 × 32.5 cm) and washed with 500 ml of 0.01 M Tris·HCl buffer, and the proteins were eluted with two Tris·HCl linear gradients (1.25 liters of 0.01 M Tris·HCl and 1.25 liters of 0.2 M Tris·HCl, 1.25 liters of 0.2 M Tris·HCl and 1.25 liters of 0.4 M Tris·HCl). The hydrogenase was collected in approximately 10-ml fractions between 2.2 and 3 liters.

Hydroxylapatite column. The collected hydrogenase-containing fractions were loaded onto a hydroxylapatite (Bio-Rad) column (4.5 × 24.5 cm) and the column was washed with 100 ml of 1 mM potassium phosphate (KP) buffer at a flow rate of 40 ml per hour. The protein was then eluted by two phosphate linear gradients (1.25 liters of 1 mM KP buffer and 1.25 liters of 0.2 M KP buffer, 625 ml of 0.2 M KP buffer and 625 ml of 0.3 M KP buffer). The hydrogenase activity was detected between 1650 ml and 2390 ml and the volume concentrated to 15 ml by using a diaflow apparatus with a YM 30 membrane.

Second DEAE-Bio-Gel column. The concentrated protein solution was diluted 1:10 with 10 mM Tris·HCl and then applied to the second DEAE-Bio-Gel column (4.5 × 22.5 cm). The column was washed with 200 ml of 0.01 M Tris·HCl, and then a linear gradient was constructed (750 ml of 0.01 M Tris·HCl and 750 ml of 0.2 M Tris·HCl). The hydrogenase was collected between 1200 and 1330 ml and the volume was concentrated to 14 ml.

Sephacryl S-200 column. The protein was loaded on a Sephacryl S-200 column (5.4 × 85 cm) and eluted with 0.05 M KP buffer at a flow rate of 20 ml/hr. The protein was collected in 5 ml-fractions between 650 and 700 ml. Purity of the hydrogenase was established by polyacrylamide disc electrophoresis (10) as well as $NaDodSO_4$/acrylamide electrophoresis (11). The purified hydrogenase had a specific activity of 4800 μmol of H_2/min per mg of protein and an $A_{400\ nm}/A_{280\ nm}$ ratio of 0.36.

Assays and Metal Determination. Hydrogenase activity was determined by the H_2 evolution assay (12). Hydrogen was determined by means of a Varian 4600 gas chromatograph (4) and protein, by the Bradford method (13) using bovine serum albumin as a reference standard. Metal content was determined by plasma emission spectroscopy using a Jarrell-Ash model 750 atomcomp.

EPR and Mössbauer Methods. EPR measurements were made with a Varian E-109 spectrometer. The Mössbauer spectrometer is of the constant-acceleration type. Both spectrometers have been described (14). The centroid of room temperature Mössbauer spectra of a metallic iron foil was taken as zero velocity.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: DCIP, dichlorophenolindophenol.

Table 1. Purification of hydrogenase from *D. vulgaris*

Fraction	Vol, ml	Protein, mg	Activity: Total, μmol H_2/min	Activity: Specific, μmol H_2/min per mg	Recovery, %
Crude extract	455	1.45×10^4	1.20×10^6	83	100
1st DEAE-Bio-Gel column	785	4.04×10^3	9.95×10^5	246	83
Hydroxylapatite column	660	516	6.04×10^5	1.45×10^3	50
2nd DEAE-Bio-Gel column	14.0	99	3.92×10^5	3.95×10^3	33
Sephacryl S-200 column	1.9	54	2.39×10^5	4.80×10^3	20

RESULTS

Metal Content. Plasma emission spectroscopy was used to determine the metal content of three different hydrogenase preparations. An iron concentration of 11 (±1) iron atoms per mol of purified hydrogenase was found; however, no nickel was detected above blank values. The nickel/iron ratio (by weight) was <0.01. In addition, EPR spectroscopy at temperatures between 10 and 100 K did not reveal any EPR resonances that could be attributed to a paramagnetic form of nickel.

EPR and Mössbauer Studies. *Native hydrogenase.* A Mössbauer spectrum of native *D. vulgaris* hydrogenase (as purified) is shown in Fig. 1. The data were recorded at 4.2 K with a magnetic field of 500 G applied parallel to the γ-beam. The spectrum consists of a broad quadrupole doublet. The apparent quadrupole splitting (ΔE_Q = 1.25 mm/s), the isomer shift (δ = 0.42 mm/s), and the shape of the spectrum are typical of $[4Fe-4S]^{2+}$ clusters. For comparison, a theoretical spectrum of *Bacillus stearothermophilus* ferredoxin (15) is also shown in Fig. 1. The close resemblance between spectra A and B in Fig. 1 suggests that the majority of the iron atoms in purified *D. vulgaris* hydrogenase are organized in the form of $[4Fe-4S]^{2+}$ clusters.

Purified hydrogenase exhibits a weak isotropic EPR signal at g = 2.02 that is similar to that found with a 3Fe cluster. Spin quantitation of this signal in various isolated native hydrogenases indicates recoveries ranging from 0.05 to 0.15 spin per molecule of hydrogenase. However, the Mössbauer spectrum of the native enzyme shows no trace of a 3Fe cluster. This observation suggests that either the amount of 3Fe cluster present in the Mössbauer sample is insignificant or the isotropic EPR signal has a different origin.

Hydrogen-reduced hydrogenase. When *D. vulgaris* hydrogenase is kept under a hydrogen atmosphere for several (12–24) hours, a complex EPR spectrum appears. At temperatures lower than 20 K, a typical "g = 1.94" type EPR signal, indicative of a reduced ferredoxin iron–sulfur cluster, dominates the spectrum with approximate g values of 2.05, 1.93, and 1.90. In addition an axial EPR signal with g_z = 2.06, $g_x = g_y$ = 2.00 is observed (Fig. 2, spectrum A). At higher temperatures (e.g., 77 K), only the axial signal is observed (Fig. 3). For convenience, the paramagnetic species that yields the axial EPR signal is termed the g = 2.06 species. Double integration of the hydrogen-reduced EPR spectrum against a Cu(II)EDTA standard yields a spin recovery of approximately 1.4 spins per molecule for the g = 1.94 species and approximately 0.03 spin per molecule for the g = 2.06 species.

The g values of the axial EPR signal, and the fact that the signal could still be observed above 77 K, are rather unusual parameters for an iron–sulfur cluster. Also, the intensity of this g = 2.06 signal could be increased by treating the reduced enzyme with CO (unpublished results; also see below). The EPR spectra of hydrogen-reduced ^{57}Fe-enriched hydrogenase and of unenriched hydrogenase at 77 K after the exposure to CO are compared in Fig. 3. The EPR spectrum of the ^{57}Fe-enriched hydrogenase relative to that of the unenriched hydrogenase shows a broadened g = 2.06 signal. The extent of broadening in the g = 2.00 region is 12 G. This observed broadening unequivocally proves that the g = 2.06 EPR signal is derived from an iron-containing complex.

Dichlorophenolindophenol-oxidized hydrogenase. The intensity of the g = 2.06 EPR signal could be increased by about 10-fold if the hydrogen-reduced enzyme was allowed

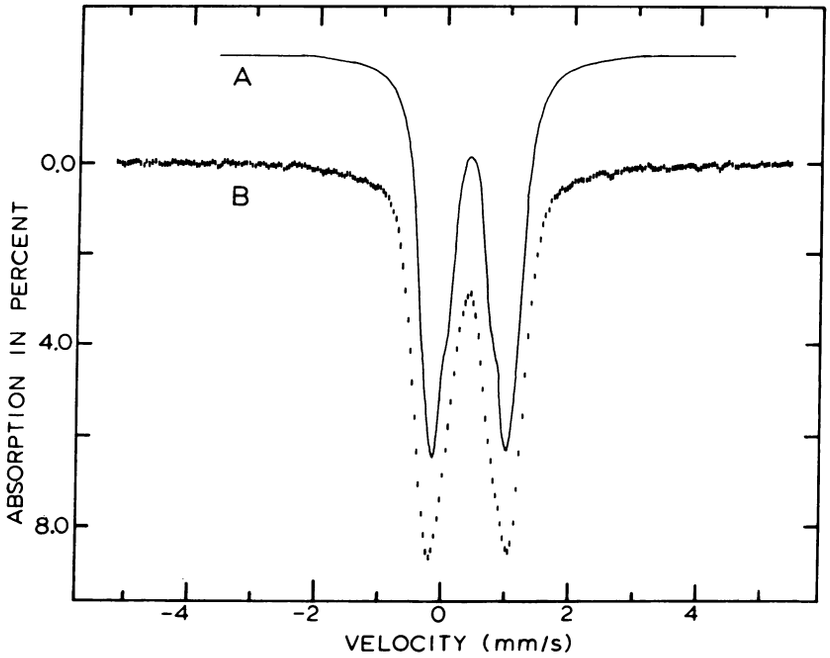

FIG. 1. Mössbauer spectrum of ^{57}Fe-enriched hydrogenase purified from *D. vulgaris*. The spectrum (trace B) was recorded at 4.2 K with a magnetic field of 500 G applied parallel to the γ-beam. The solid line (trace A) is a simulated spectrum of oxidized ferredoxin from *B. stearothermophilus*, which contains a $[4Fe-4S]^{2+}$ cluster. The parameters used for the simulation were taken from ref. 15.

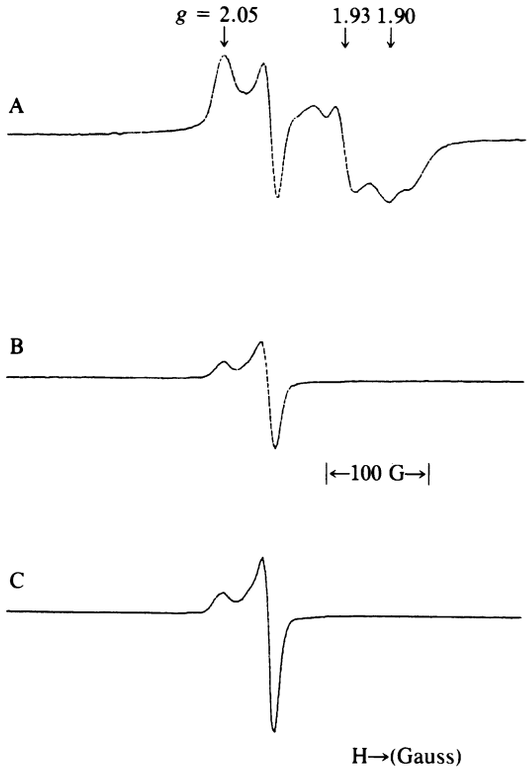

FIG. 2. EPR spectra of ^{57}Fe-enriched hydrogenase from *D. vulgaris*. The sample was 1.25 mM in protein in 50 mM potassium phosphate buffer (pH 7.6) and was contained in a combination EPR/Mössbauer holder that can be studied in both EPR and Mössbauer spectrometers. Spectra: A, sample reduced with H_2 for 48 hr; B, the hydrogen-reduced sample was treated with CO for 5 hr; C, the hydrogen-reduced sample was treated with 21 mM dichlorophenolindophenol. EPR conditions: microwave power, 20 μW; microwave frequency, 9.160 GHz; modulation amplitude, 10 G; temperature, 10 K; time constant, 0.1 s; scanning rate, 400 G/min.

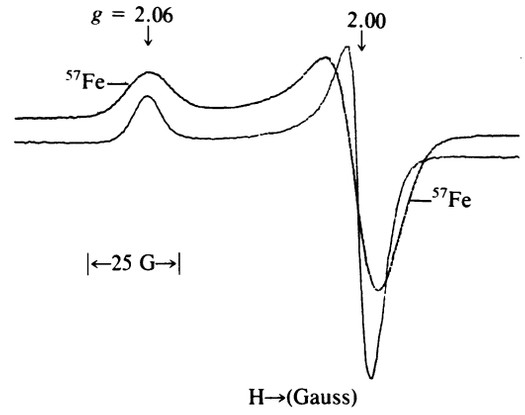

FIG. 3. Effect of ^{57}Fe isotopic substitution on EPR spectra of hydrogenase from *D. vulgaris*. ^{56}Fe hydrogenase or ^{57}Fe-enriched hydrogenase was reduced with H_2 for 16 hr and then treated with CO for 30 min. The ^{56}Fe hydrogenase was 89 μM in protein in 50 mM potassium phosphate buffer (pH 7.6) while the ^{57}Fe-enriched hydrogenase was 103 μM in protein and in the same buffer system. EPR conditions: microwave power, 10 mW; microwave frequency, 9.163 GHz; modulation amplitude, 5 G; temperature, 77 K; time constant, 0.1 s; scanning rate, 125 G/min.

to react with dichlorophenolindophenol (DCIP) or with cytochrome c_3, its physiological electron carrier. To study this g = 2.06 species in more detail, we have prepared a DCIP-oxidized Mössbauer/EPR sample that can be studied in both Mössbauer and EPR spectrometers (since cytochrome c_3 is EPR active and complicates the spectroscopic analysis, we chose DCIP for this study). The EPR spectrum is shown in Fig. 2 as trace C. The data were recorded at 10 K. Only the g = 2.06 signal was detected. Spin quantitation of this signal yields approximately 0.3 spin per molecule. The complete disappearance of the g = 1.94 signal suggests oxidation of some of the $[4Fe\text{–}4S]^{1+}$ clusters to an EPR-silent, presumably $[4Fe\text{–}4S]^{2+}$ state.

The Mössbauer spectra of the DCIP-oxidized enzyme are shown in Fig. 4. The data were recorded at 4.2 K with a magnetic field of 500 G applied parallel and perpendicular, respectively, to the γ-beam. Both spectra have at least two subspectral components: an intense quadrupole doublet at the center and a paramagnetic component (component M_A) extended from −1.7 mm/s to 2.5 mm/s. The doublet accounts for approximately 80% of the total iron resonance absorption. Its Mössbauer parameters (ΔE_Q and δ) are identical

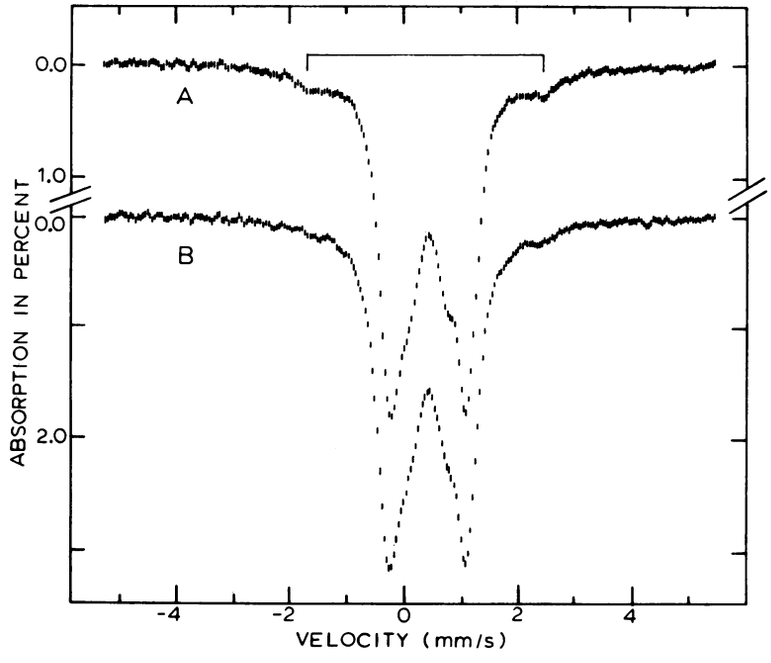

FIG. 4. Mössbauer spectra of DCIP-oxidized ^{57}Fe-enriched hydrogenase from *D. vulgaris*. Data were recorded at 4.2 K with a magnetic field of 500 G applied parallel (trace A) and perpendicular (trace B) to the γ radiation. The EPR spectrum of this sample is shown in Fig. 2 (trace C). The bracket indicates the outer-most resonance absorption of component M_A.

to that of the native hydrogenase—i.e., typical for EPR-silent $[4Fe-4S]^{2+}$ clusters.

Comparison of spectra A and B in Fig. 4 indicates that component M_A exhibits an absorption pattern that is dependent on the direction of the applied magnetic field. It has been shown that a paramagnetic species that yields field-direction-dependent Mössbauer spectra should also be EPR active (16). Since the DCIP-oxidized sample exhibits only the $g = 2.06$ EPR signal, component M_A can only be attributed to the $g = 2.06$ species. Also, at higher temperatures (77 K), component M_A remains magnetic, indicating that its electronic relaxation time is comparable with and longer than the nuclear precession time (≈10 ns). This property is in agreement with the fact that the $g = 2.06$ EPR signal can be observed at 77 K.

The magnetic splitting of component M_A is about 4.2 mm/s, which corresponds to a hyperfine field of 130 kG at the Fe nucleus and an equivalent 12.6 G at the electron. A broadening of 12 G was observed in the EPR signal of an ^{57}Fe-enriched sample (see above). The observed magnetic splitting of component M_A is similar to that of a paramagnetic 4Fe cluster (15, 17) but smaller than that of an oxidized 3Fe cluster (6, 18, 19). A rough estimate reveals that component M_A could account for 10–15% of the total iron absorption. Assuming that the enzyme contains 12 iron atoms per molecule and that the $g = 2.06$ species is a cluster containing four Fe atoms, then 10–15% of the total absorption should yield 0.3–0.4 spin per molecule, in reasonable agreement with the EPR findings.

Taken together, the aforementioned observations and the fact that DCIP and cytochrome c_3 are electron acceptors, we tentatively consider the $g = 2.06$ species to be an atypical $[4Fe-4S]^{3+}$ cluster. However, considering the uncertainties in estimating the percentage iron resonance absorption, the iron content, and the spin concentration, such an assignment has to be viewed as provisional. Also, in view of the unusual g values and the electronic relaxation properties, we do not exclude the possibility that the $g = 2.06$ species may be a novel iron cluster.

CO-treated hydrogenase. When the hydrogen-reduced enzyme was allowed to react with CO, the $g = 2.06$ signal was observed to increase to the same intensity as found with cytochrome c_3 or DCIP treatment of hydrogen-reduced enzyme. However, in some cases the $g = 1.94$ signal was still detectable, although its intensity was much reduced in comparison with that of hydrogen-reduced enzyme. Again, for more detailed studies, a Mössbauer/EPR sample of CO-treated hydrogenase was prepared. The EPR spectrum is shown in Fig. 2 (trace B) and the Mössbauer spectrum is shown in Fig. 5. The Mössbauer spectrum shows the familiar central quadrupole doublet with parameters similar to those of an $[4Fe-4S]^{2+}$ cluster. The peaks at −1.7 mm/s and +2.5 mm/s represent absorptions originating from the $g = 2.06$ species. In addition, a novel paramagnetic component (component M_B) is observed with resonance absorption extended from −4 mm/s to +6 mm/s. Such a large magnetic splitting is unusual for an Fe–S cluster. Moreover, component M_B exhibits no field-direction dependence, indicating that the electronic state associated with component M_B should yield no EPR resonance. This unique electronic property can be explained by an electronic system of half-integral spin ($S \geq 3/2$) with a negative zero-field-splitting parameter D. For iron clusters, such an electronic system has been detected only for the oxidized P cluster in nitrogenase (20). Thus, in addition to the $g = 2.06$ species, there is yet another atypical cluster in hydrogenase, which is represented by component M_B. Since component M_B appears only in CO-treated hydrogenase, it is likely that component M_B may represent a CO-bound iron cluster.

DISCUSSION

The present studies establish that the hydrogenase from *D. vulgaris* contains no detectable nickel and that the majority of the iron atoms in *D. vulgaris* hydrogenase are bound in the form of iron–sulfur clusters. In the enzyme as prepared, two ferredoxin-type [4Fe–4S] clusters have been identified that exhibit normal EPR and Mössbauer parameters. In addition, two novel states for iron clusters were discovered in this enzyme. One is the species that yields the magnetic Mössbauer component M_B, presumably a CO-bound species; the other is the $g = 2.06$ species, which is enhanced by reaction of cytochrome c_3, DCIP, or CO with the reduced hydrogenase and exhibits temperature characteristics unique for an iron–sulfur cluster.

On the basis of function, hydrogenases have been categorized into two types, "uptake" and "evolution," and it has been suggested that uptake hydrogenases can be characterized as containing nickel (21). Our results show conclusively

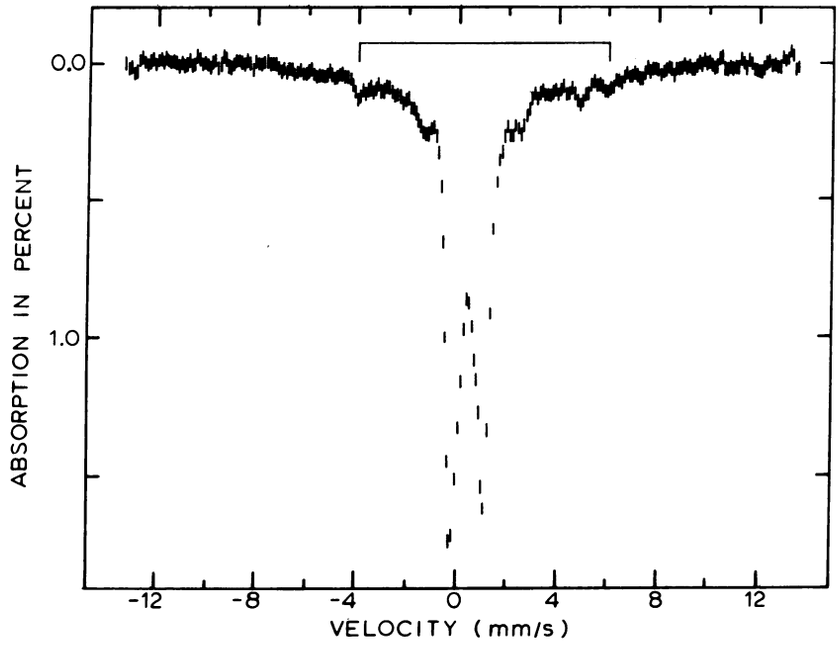

FIG. 5. Mössbauer spectrum of ^{57}Fe-enriched hydrogenase from *D. vulgaris* after CO treatment. Data were recorded at 4.2 K with a 500-G parallel field. The EPR spectrum of this sample is shown in Fig. 2 (trace B). The bracket indicates the extent of the Mössbauer resonance absorption of component M_B.

that the periplasmic hydrogenases and uptake hydrogenases found in bacteria belonging to the genus *Desulfovibrio* are of two types—the nickel- and 3Fe cluster-containing hydrogenases found in *D. gigas* (4) and *D. desulfuricans* (6) and the hydrogenase lacking nickel and the 3Fe cluster found in *D. vulgaris* that is similar to the evolution hydrogenases of *C. pasteurianum* and *M. elsdenii*.

The $g = 2.06, 2.00$ EPR signal has been observed in hydrogenase purified from *C. pasteurianum* after the reduced enzyme had been allowed to react with CO (22). EPR investigation using 90% enriched ^{13}CO to replace ^{12}CO induced a slight broadening of the $g = 2.06$ signal. Based on this observation, Erbes *et al.* (22) suggested that the $g = 2.06$ signal represents a CO-bound iron–sulfur cluster. We have made EPR measurements on ^{13}CO-treated hydrogenase of *D. vulgaris*. A broadening of 1.3 G was detected at the $g = 2.00$ region for the $g = 2.06, 2.00$ signal; however, this slight broadening may well be within the experimental limitation of our equipment. The present studies show that this signal does appear in the *absence* of CO; thus, the signal is observed in hydrogen-reduced hydrogenase, and its intensity can be increased to about ten-fold by allowing the reduced enzyme to react with either DCIP or oxidized cytochrome c_3. Consequently, this signal cannot represent a CO-bound species.

Van Dijk *et al.* (23) have reported the presence of the $g = 2.06$ species in the purified hydrogenase (reduced) from *M. elsdenii* and suggested that the $g = 2.06$ signal is due to irreversible inactivated enzyme. In the following, we list some observations suggesting that the $g = 2.06$ signal may have physiological significance and not represent oxidatively damaged protein. The $g = 2.06$ signal is commonly observed in hydrogenase. It has previously been observed in hydrogenases purified from *C. pasteurianum* (22) and *M. elsdenii* (23), and now it has been observed in *D. vulgaris*. In the case of *D. vulgaris* hydrogenase, this signal is observed in the hydrogen-reduced enzyme, in the CO-treated enzyme, and in the presence of cytochrome c_3, the natural electron carrier for this hydrogenase (24). It should be noted that cytochrome c_3 does not inhibit hydrogenase and that inhibition by CO of the hydrogen-evolution activity is completely reversible.

Grande *et al.* (8) have reported that their hydrogenase purified from *D. vulgaris* contained three $[4Fe\text{–}4S]^{2+,1+}$ clusters of the bacterial ferredoxin type; however, one of the clusters was not stable to redox cycling with ferricyanide and dithionite and was termed a "special cluster." It is not yet clear whether there exists any relationship between this special cluster and the observations presented in this communication. The structure and function of these abnormal iron–sulfur clusters are being further investigated.

We thank Susan Benton and Liesje DerVartanian for excellent technical assistance with the computer-assisted EPR spectroscopy and Professor John Wampler for interfacing and programming the Varian E-109 spectrometer with the Hewlett-Packard Model 85 microcomputer. We thank the staff of the fermentation plant for growing the bacterial cells. This study was supported in part by National Science Foundation Grants PCM-8111325 to J.L., D.V.D., and H.D.P., Jr.; PCM-8108092 to B.H.H.; and PCM-8213874 to D.V.D. This research was also supported by Solar Energy Research Institute Grant XD-1-11-551 to J.L., National Institutes of Health Grants AM01135-01 and GM 32187-01 to B.H.H., and Department of Energy Grant DEA 509-79ER10499-A002 to H.D.P., Jr.

1. Van der Westen, H. M., Mayhew, S. G. & Veeger, G. (1978) *FEBS Lett.* **86,** 122–126.
2. Chen, J. S., Mortenson, L. E. & Palmer, G. (1976) in *Iron and Copper Proteins*, eds. Yasunobu, K. T., Mower, H. F. & Hayaishi, O. (Plenum, New York), pp. 68–82.
3. Van Dijk, C., Mayhew, S. G., Grande, H. J. & Veeger, C. (1979) *Eur. J. Biochem.* **102,** 317–330.
4. LeGall, J., Ljungdahl, P. O., Moura, I., Peck, H. D., Jr., Xavier, A. V., Moura, J. J. G., Teixeira, M., Huynh, B. H. & DerVartanian, D. V. (1982) *Biochem. Biophys. Res. Commun.* **106,** 610–616.
5. Cammack, R., Patil, D., Aguirre, R. & Hatchikian, C. (1982) *FEBS Lett.* **142,** 289–292.
6. Krüger, H. J., Huynh, B. H., Ljungdahl, P. O., Xavier, A. V., DerVartanian, D. V., Moura, I., Peck, H. D., Jr., Teixeira, M., Moura, J. J. G. & LeGall, J. (1982) *J. Biol. Chem.* **257,** 14620–14623.
7. Hatchikian, E. C., Bruschi, M. & LeGall, J. (1948) *Biochem. Biophys. Res. Commun.* **82,** 451–461.
8. Grande, H. J., Dunham, W. R., Averill, B., Van Dijk, C. & Sands, R. H. (1983) *Eur. J. Biochem.* **136,** 201–207.
9. Wolin, E. A., Wolfe, R. S. & Wolin, M. J. (1964) *J. Bacteriol.* **87,** 993–998.
10. Brewer, J. M. & Ashworth, R. B. (1969) *J. Chem. Educ.* **46,** 41–45.
11. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10,** 2602–2617.
12. Peck, H. D., Jr., & Gest, H. (1956) *J. Bacteriol.* **71,** 70–80.
13. Bradford, M. N. (1976) *Anal. Biochem.* **72,** 248–254.
14. Huynh, B. H., Lui, M. C., Moura, J. J. G., Moura, I., Ljungdahl, P. O., Münck, E., Payne, W. J., Peck, H. D., Jr., DerVartanian, D. V. & LeGall, J. (1982) *J. Biol. Chem.* **257,** 9576–9581.
15. Middleton, P., Dickson, D. P. E., Johnson, C. E. & Rush, J. D. (1978) *Eur. J. Biochem.* **88,** 135–141.
16. Huynh, B. H. & Kent, T. A. (1983) in *Advances in Mössbauer Spectroscopy Applications to Physics, Chemistry and Biology*, eds. Thosar, V. B., Iyengar, P. K., Sirvastava, J. K. & Bhargava, S. C. (Elsevier Scientific Publishing, Amsterdam), pp. 490–560.
17. Middleton, P., Dickson, D. P. E., Johnson, C. E. & Rush, J. D. (1980) *Eur. J. Biochem.* **104,** 289–296.
18. Emtage, M. H., Kent, T. A., Huynh, B. H., Rawlings, J., Orme-Johnson, W. H. & Münck, E. (1980) *J. Biol. Chem.* **255,** 1793–1796.
19. Huynh, B. H., Moura, J. J. G., Moura, I., Kent, T. A., LeGall, J., Xavier, A. V. & Münck, E. (1980) *J. Biol. Chem.* **255,** 3242–3244.
20. Huynh, B. H., Henzl, M. T., Christner, J. A., Zimmermann, R., Orme-Johnson, W. H. & Münck, E. (1980) *Biochim. Biophys. Acta* **623,** 124–138.
21. Unden, G., Böcher, R., Knecht, J. & Kröger, A. (1982) *FEBS Lett.* **145,** 230–234.
22. Erbes, D. L., Burris, R. H. & Orme-Johnson, W. H. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4795–4799.
23. Van Dijk, G., Grande, H. J., Mayhew, S. G. & Veeger, C. (1980) *Eur. J. Biochem.* **107,** 251–261.
24. Yagi, T. (1970) *J. Biochem. (Tokyo)* **68,** 649–657.