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EPR-detectable Redox Centers of the Periplasmic Hydrogenase from *Desulfovibrio vulgaris**

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The periplasmic hydrogenase of *Desulfovibrio vulgaris* (Hildenbourough NCIB 8303) belongs to the category of [Fe] hydrogenase which contains only iron-sulfur clusters as its prosthetic groups. Amino acid analyses were performed on the purified *D. vulgaris* hydrogenase. The amino acid composition obtained compared very well with the result derived from the nucleotide sequence of the structural gene (Voordouw, G., Brenner, S. (1985) *Eur. J. Biochem.* 148, 515–520). Detailed EPR reductive titration studies on the *D. vulgaris* hydrogenase were performed to characterize the metal centers in this hydrogenase. In addition to the three previously observed EPR signals (namely, the "isotropic" 2.02 signal, the rhombic 2.10 signal, and the complex signal of the reduced enzyme), a rhombic signal with resonances at the *g*-values of 2.06, 1.96, and 1.89 (the rhombic 2.06 signal) was detected when the samples were poised at potentials between 0 and −250 mV (with respect to normal hydrogen electrode). The midpoint redox potentials for each of the four EPR-active species were determined, and the characteristics of each EPR signal are described. Both the rhombic 2.10 and 2.06 signals exhibit spectral properties that are distinct from a ferredoxin-type [4Fe-4S] cluster and are proposed to originate from the same H_2-binding center but in two different conformations. The complex signal of the reduced hydrogenase has been shown to represent two spin-spin interacting ferredoxin-type $[4Fe\text{-}4S]^{1+}$ clusters (Grande, H. J., Dunham, W. R., Averill, B., Van Dijk, C., and Sands, R. H. (1983) *Eur. J. Biochem.* 136, 201–207). The titration data indicated a strong cooperative effect between these two clusters during their reduction.

In an effort to accurately estimate the number of iron atoms/molecule of hydrogenase, plasma emission and chemical methods were used to determine the iron contents in the samples; and four different methods, including amino acid analysis, were used for protein determination. The resulting iron stoichiometries were found to be method-dependent and vary over a wide range (±20%). The uncertainties involved in the determination of iron stoichiometry are discussed.

Hydrogenases are a class of enzymes that catalyze the oxidation of molecular hydrogen and/or the reduction of protons. They are a heterogeneous group of enzymes that differ in molecular composition, metal content, and specific activity (Adams *et al.*, 1981; LeGall *et al.*, 1982); and evidence for the existence of multiple hydrogenases within one organism has been repeatedly reported (Lissolo *et al.*, 1986; Teixeira *et al.*, 1987). Based on their metal contents, however, hydrogenases may be grouped into three categories: 1) the [NiFe] hydrogenases, which contain both nickel and iron atoms; 2) the [NiFeSe] hydrogenases, which contain nickel, iron, and selenium atoms; and 3) the [Fe] hydrogenases, which contain only iron atoms. In assays with artificial electron donors or acceptors, the [Fe] hydrogenases generally exhibit much higher specific activities than those hydrogenases containing nickel.

The periplasmic hydrogenase of *Desulfovibrio vulgaris* (Hildenbourough NCIB 8303) belongs to the [Fe] hydrogenase category. Other [Fe] hydrogenases can also be found in anaerobic bacteria of the genera *Clostridium* (Chen and Mortenson, 1974), *Acetobacterium* (Ragsdale and Ljungdahl, 1984), and *Megasphaera* (Van Dijk *et al.*, 1979). The nucleotide sequence of the structural gene for the *D. vulgaris* hydrogenase indicates that this hydrogenase is composed of two subunits with molecular masses of 45.8 and 13.5 kDa (Voordouw and Brenner, 1985). Sodium dodecyl sulfate gel electrophoresis of the isolated enzyme also showed a two-band pattern corresponding to two apparent molecular masses of 46 and 13.5 kDa (Hagen *et al.*, 1986a). In comparison with the gene-encoded sequence, amino acid sequencing studies of the purified hydrogenase, however, indicated that the small subunit lacks a hydrophobic NH_2-terminal amino acid sequence, which has general characteristics of a signal peptide (Prickril *et al.*, 1986). The molecular mass of the small subunit excluding the signal peptide was estimated to be 10 kDa.

Determination of the metal contents using plasma emission spectroscopy has firmly established that *D. vulgaris* hydrogenase contains only iron atoms (Huynh *et al.*, 1984). The number of iron atoms/molecule reported in the literature varies from 10 to 16 atoms/molecule (Huynh *et al.*, 1984; Hagen *et al.*, 1986a). Comparable numbers of sulfur atoms were also reported with a similar range of variation. These variations in iron and sulfur contents are largely due to the difficulties involved in determining protein concentration (Hagen *et al.*, 1986a). The derived amino acid sequence (Voordouw and Brenner, 1985) revealed that the large subunit

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contained a region (residues 26–85) that is homologous to the bacterial 8-iron ferredoxins (Adman *et al.*, 1973), suggesting that 8 of the iron atoms in *D. vulgaris* hydrogenase may be organized into two ferredoxin-type [4Fe-4S] cubane clusters. The remaining iron atoms were suggested to also form a third iron-sulfur cluster, which was believed to be the substrate-binding site. It exhibited atypical spectroscopic properties that were distinctively different from a ferredoxin-type [4Fe-4S] cluster. Due to the uncertainty in the iron content, the number of the iron atoms associated with this atypical cluster can vary from 3 to 6. Cluster extrusion experiments (Grande *et al.*, 1983), however, showed that three ferredoxin-type [4Fe-4S] clusters were extruded from each molecule of *D. vulgaris* hydrogenase. Consequently, this atypical cluster is likely to be structurally related to the [4Fe-4S] cubane cluster and able to convert into a normal [4Fe-4S] cluster under the extrusion conditions.

To characterize the metal centers in *D. vulgaris* hydrogenase, an EPR reductive titration study was carried out in our laboratory. In this manuscript, the results of this study are presented, and the characteristics of each EPR signal are described. The midpoint redox potential and the maximum spin concentration attained for each EPR-active species are reported. The iron content of the *D. vulgaris* hydrogenase has also been re-evaluated using amino acid composition for protein determination.

MATERIALS AND METHODS

Purification of Hydrogenase—*D. vulgaris* hydrogenase was purified by the procedure described previously (Huynh *et al.*, 1984) with the following modifications. A DE52 column (6 × 32.5 cm) was used instead of the first DEAE-Bio-Gel column, and the second DEAE-Bio-Gel column was found unnecessary. After the gel filtration column (Sephacryl S-200), the enzyme preparation was further purified with a Beckman preparative HPLC[1] system with an ion-exchange column (TSK DEAE-3SW). The purity of the protein was checked by sodium dodecyl sulfate gel electrophoresis, which showed only the two subunit bands; and no trace of impurities was detected. The specific activity of the protein sample in the hydrogen evolution assay was 4000 ± 200 μmol of H_2/min/mg of protein.

Amino Acid Composition Determination—The amino acid composition was determined by hydrolysis of the protein sample with 6 N HCl for 24, 48, and 72 h at 110 °C in vacuum-sealed Pyrex test tubes. Norleucine was used as an internal marker to establish the recovery of the amino acids after acid hydrolysis. Cysteine was determined as cysteic acid after performic acid oxidation (Hirs, 1967). Tryptophan was determined after hydrolysis in the presence of 2% thioglycolic acid (Matsubara and Sasaki, 1969). Phenyl isothiocyanate was used for the quantitative precolumn derivatization of amino acids with Pierce Amino Acid Standard H as standard. The phenyl isothiocyanate-derivatized samples were analyzed by a reverse-phase column (Altex ODS; 4.6 × 150 mm) using a Beckman HPLC system 334 (Heinrikson and Meridth, 1984).

Iron and Protein Determination—Iron content was determined both by plasma emission spectroscopy using a Jarrell-Ash Model 750 Atomcomp instrument and by the chemical method (Massey, 1957), in which the formation of ferrous *o*-phenanthrolinate is measured after trichloroacetic acid precipitation of the protein. Amino acid analysis was used to estimate the amount of protein in the samples. For comparison, protein concentrations were also determined by the Bradford (1976), biuret (Gornall *et al.*, 1949), and Lowry (Lowry *et al.*, 1951) methods using bovine serum albumin as standard.

Reductive Titration—The redox titrations were carried out at 25 °C in a titration assembly similar to the one described by Dutton (1978). The protein solution (50 μM) was in a 200 mM Tris-HCl buffer (pH 7.0). The cell, equipped with a platinum and a calomel standard electrode, was calibrated with a saturated solution of quinhydrone at pH 7 and cross-checked with an equimolar solution of potassium ferricyanide/potassium ferrocyanide. During the titrations, the system was kept anaerobic by a constant purge of oxygen-free argon gas, which had been passed through a heated copper catalyst and sodium dithionite solution. The mediator dyes (40 μM each) used in the titration were phenazine ethosulfate (+55 mV),[2] methylene blue (+11 mV), resorufin (−51 mV), indigo disulfonate (−125 mV), 2-hydroxy-1-4-naphthaquinone (−145 mV), anthraquinone 2-sulfonate (−225 mV), phenosafranin (−252 mV), safranin O (−280 mV), neutral red (−340 mV), benzyl viologen (−350 mV), and methyl viologen (−440 mV). Sodium dithionite solution (100 mM) was used as the reductant. After each addition of the reductant, the protein solution was allowed to stabilize for ~5 min before a sample was withdrawn with a gas-tight syringe and transferred into an EPR tube which was attached to the redox vessel. The sample in the EPR tube was quickly frozen before the tube was detached from the vessel for EPR measurements. The potentials are reported with respect to the normal hydrogen electrode and have an estimated uncertainty of ±15 mV (Dutton, 1978). The cell potentials were measured with respect to the saturated calomel electrode potential of +247 mV (Clark, 1972).

EPR—EPR measurements were performed on a Bruker ER 200D-SRC spectrometer equipped with an Oxford Instruments ESR 910 continuous flow cryostat. The data were recorded in a Bruker Aspect 2000 computer and transferred to an IBM AT computer for data analysis. EPR spin quantitations were performed using CuEDTA (1 mM) and myoglobin azide (220 μM, 50 mM phosphate buffer (pH 7.6)) as standards and applying the procedures described by Aasa and Vängård (1975).

RESULTS

Amino Acid Composition and Iron Content Determination

Three separate amino acid analyses were performed on the purified *D. vulgaris* hydrogenase, and the results are listed in Table I. For comparison, the amino acid composition derived from the nucleotide sequence of the structural gene (Voordouw and Brenner, 1985), excluding the signal peptide, is also tabulated. The good agreement between these two results indicated that our purification procedure yielded very pure proteins.

Based on the amino acid analysis, we have re-evaluated the extinction coefficient and found $\varepsilon_{400} = 48.3\ mM^{-1}\ cm^{-1}$, which was comparable to, but different from, the previously reported value of 45 $mM^{-1}\ cm^{-1}$ (Hagen *et al.*, 1986a). The previous value was calculated based on a molecular mass of 59.5 kDa, which is an overestimate since it included the mass of the signal peptide segment in the nucleotide gene sequence. Excluding the signal peptide, the correct molecular mass should be 56 kDa.

For iron content determination, four different preparations of *D. vulgaris* hydrogenase were used, and eight separate measurements were made. Amino acid analyses were used for determining the amount of proteins in three of the eight measurements. For the remaining measurements, proteins were estimated spectrophotometrically using the extinction coefficient $\varepsilon_{400} = 48.3\ mM^{-1}\ cm^{-1}$. The iron contents so determined were found to have an average value of 10 ± 1 iron atoms/molecule of molecular mass of 56 kDa. To examine the effect of protein determination methods on the value of iron content, the Bradford (1976), biuret (Gornall *et al.*, 1949), and Lowry (Lowry *et al.*, 1951) methods were also used for protein determinations for a given sample. The results are listed in Table II and are compared with the results derived from amino acid analysis. The protein concentration varies depending on the method used: the Bradford method tends to underestimate by about 20%, whereas the biuret and Lowry methods tend to overestimate by a similar amount in comparison with the value obtained from the amino acid analysis. The iron content consequently varies correspondingly. These data clearly demonstrate that the iron stoichiometry derived

[1] The abbreviations used are: HPLC, high performance liquid chromatography: W, watt(s).

[2] The values given in parentheses are the oxidation-reduction midpoint potentials of the mediators at pH 7.

TABLE I
Amino acid composition of D. vulgaris hydrogenase

Residue	Amino acid analysis[a]	Nucleotide sequence[b]
	residues/mol	*residues/mol*
Lys	46	47
His	13	13
Arg	21	18
Asp + Asn	35	29 + 8
Thr	33	32
Ser	17	23
Glu + Gln	48	33 + 13
Pro	31	32
Gly	44	45
Ala	48	49
Cys	17	18
Val	32	32
Met	19	19
Ile	26	21
Leu	35	32
Tyr	18	22
Phe	19	17
Trp	7	6
Total	509	509

[a] Calculation based upon a molecular mass of 56 kDa.
[b] Data derived from the nucleotide sequence of the structural gene (Voordouw and Brenner, 1985).

TABLE II
Effect of protein determination method on the iron stoichiometry

Method of protein determination	Protein content	No. Fe atoms/molecule[a]
	mg/ml	
Bradford (1976)	0.20	13.5
	0.22	12.0
Lowry *et al.* (1951)	0.32	8.5
Biuret (Gornall *et al.*, 1949)	0.29	9.2
Amino acid analysis	0.26	10.3

[a] The molecular mass was assumed to be 56 kDa, and the iron content was determined by plasma emission spectroscopy.

from various methods of protein assay contains large uncertainties. The reliable protein determination is by amino acid analysis.

EPR

Characteristic EPR spectra of *D. vulgaris* hydrogenase observed during the reductive titrations are shown in Fig. 1. The development of these spectra at selected potentials is presented in Fig. 2. The relative EPR intensities of these signals as a function of the potential are plotted in Fig. 3. Each EPR signal is described in detail below.

"Isotropic" 2.02 Signal—As isolated, *D. vulgaris* hydrogenase exhibits a nearly isotropic EPR signal at the $g = 2.02$ region (Fig. 1, *trace A*). The shape of this isotropic signal, which shows a peak at $g = 2.02$ and a derivative-type signal at $g = 2.00$, is very similar to the one found for a [3Fe-4S] cluster in *Desulfovibrio gigas* ferredoxin II (Huynh *et al.*, 1980). Spin quantitation of this isotropic 2.02 signal yields approximately 0.2 spin/molecule; and, for various hydrogenase preparations, quantitations of 0.02–0.2 spin/molecule have been reported (Grande *et al.*, 1983; Huynh *et al.*, 1984). Due to the variable and substoichiometric amount observed for this isotropic 2.02 signal and the fact that [4Fe-4S] clusters can be converted into [3Fe-4S] clusters under oxidizing conditions (Thomson *et al.*, 1981; Moura *et al.*, 1982), the presence of this signal in the isolated enzymes could represent an artifact due to the oxidation of a [4Fe-4S] cluster. During the titration, the intensity of this isotropic 2.02 signal decreased with decreasing potential (Fig. 3). The estimated midpoint redox potential is −80 mV, which is within the range of those found for [3Fe-4S] clusters (Huynh *et al.*, 1980; Teixeira *et al.*, 1985).

Rhombic 2.06 Signal—During the course of reductive titration, a rhombic EPR signal with resonances at $g = 2.06$, 1.96, and 1.89 (Fig. 1, *trace B*) appeared at about 0 mV. (see Figs. 2 and 3). The intensity of this rhombic 2.06 signal increased with decreasing redox potential and reached its maximum at about −110 mV. Below −200 mV, the signal intensity began to decrease and completely vanished below −300 mV. The midpoint potentials were determined to be −20 mV for the development of this rhombic 2.06 signal and −230 mV for the disappearance of the signal. At its maximum intensity, the signal represented approximately 0.7 spin/molecule.

This rhombic 2.06 signal can be observed at temperatures up to 40 K. The 40 K spectrum was found to be as sharp as the spectrum recorded at 8 K. At temperatures above 80 K, however, this rhombic 2.06 signal was broadened beyond detection. We have also measured the power saturation behavior of this rhombic 2.06 signal, and the results are shown in Fig. 4. We found that the signal could be easily saturated at low temperature. At 8 K, the P_{50} for this signal was determined to be 40 μW, which was significantly smaller than those found for the ferredoxin-type $[4Fe\text{-}4S]^{1+}$ clusters (on the order of 1 mW) and was similar to those of the reduced [2Fe-2S] clusters (Rupp *et al.*, 1978).

Rhombic 2.10 Signal—At −200 mV, when the intensity of the rhombic 2.06 signal began to decrease, another rhombic signal with resonances at $g = 2.10$, 2.04, and 2.00 started to appear (Fig. 2). The intensity of this rhombic 2.10 signal reached its maximum at approximately −300 mV (Fig. 3). Spin quantitation of the signal at its maximum intensity indicated a spin concentration of approximately 0.4 spin/molecule. The midpoint redox potential was determined to be −270 mV. Below −320 mV, this rhombic 2.10 signal abruptly disappeared (Fig. 3). Power saturation study indicated that this signal could also be easily saturated at low temperature. The P_{50} for this signal was determined to be 25 μW at 8 K (Fig. 4).

Complex EPR Signal of Reduced Hydrogenase—*D. vulgaris* hydrogenase can be reduced by dithionite or by incubation under hydrogen atmosphere. In the fully reduced state, *D. vulgaris* hydrogenase exhibits a complex EPR signal (Fig. 1, *trace D*), which was similar to that observed for the reduced 8-iron ferredoxin from *Micrococcus lactilyticus* (Mathews *et al.*, 1974). The complexity of the 8-iron ferredoxin spectrum was shown to be caused by the spin-spin interaction between the two $[4Fe\text{-}4S]^{1+}$ clusters. EPR measurements performed with different microwave frequencies (Grande *et al.*, 1983) demonstrated that the complex signal of the reduced *D. vulgaris* hydrogenase was also the result of spin-spin interaction. The EPR data of the reduced enzyme are consistent with the result deduced from the Mössbauer data (Huynh *et al.*, 1984), the amino acid sequence (Voordouw and Brenner, 1985), and the cluster extrusion experiment (Grande *et al.*, 1983), which indicate that the *D. vulgaris* hydrogenase contains two ferredoxin-type [4Fe-4S] clusters. In the reduced enzyme, both clusters are reduced to the $[4Fe\text{-}4S]^{1+}$ state and are spin-spin interacting to yield the complex EPR signal.

During the reductive titrations, the complex EPR signal appeared at potentials below −250 mV and was fully developed at −350 mV (Figs. 2 and 3). The complexity of the signal was partially masked by the intense radical signals generated

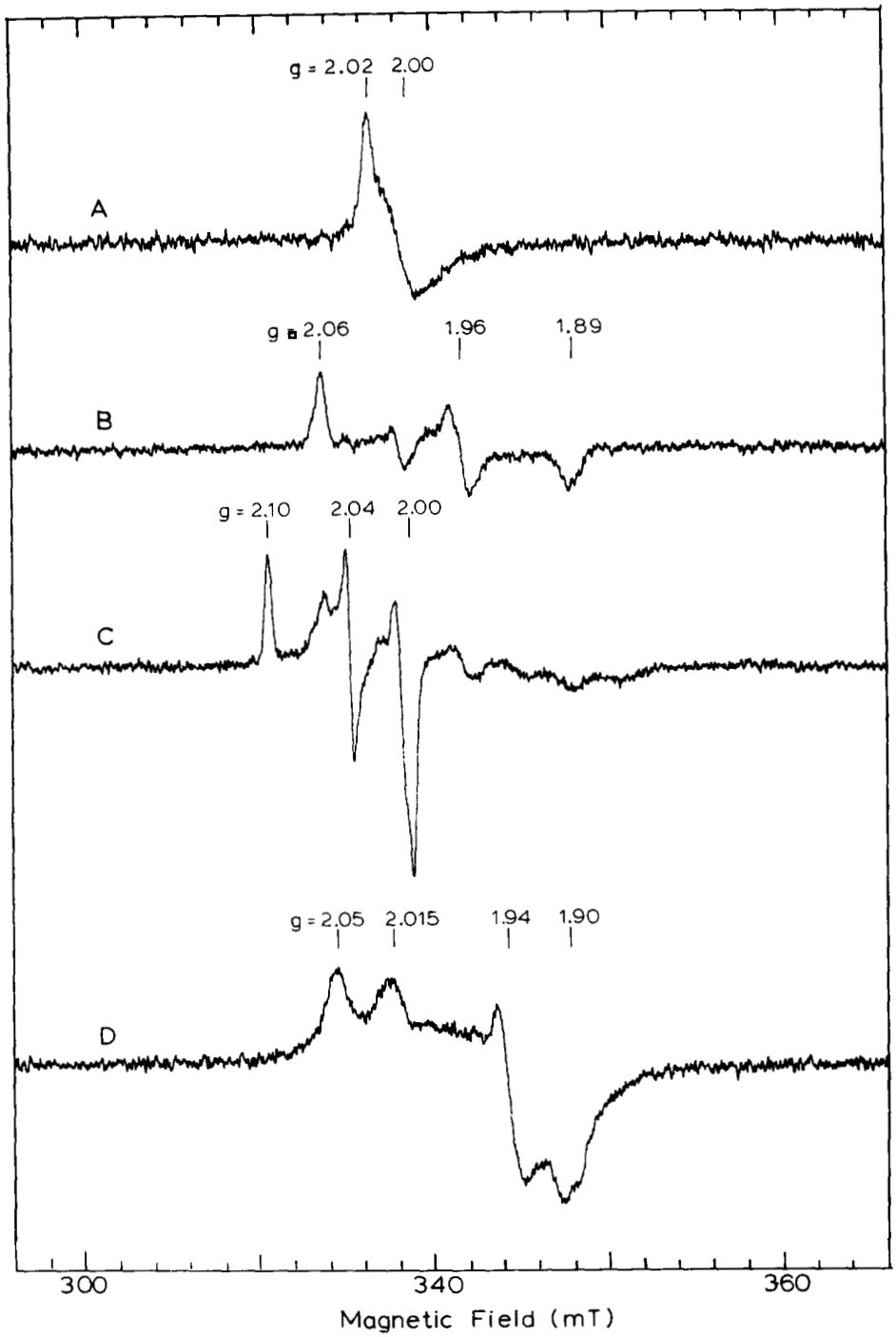

FIG. 1. **Characteristic EPR spectra of *D. vulgaris* hydrogenase at different redox states.** *Trace A*, the isotropic 2.02 signal observed in the as-isolated sample; *trace B*, the rhombic 2.06 signal observed at −160 mV; *trace C*, the rhombic 2.10 signal observed at −255 mV; *trace D*, the complex signal of the hydrogen-reduced hydrogenase. Experimental conditions were: temperature, 8 K; microwave frequency, 9.43 GHz; microwave power, 2 μW; modulation amplitude, 1.0 millitesla (*mT*); receiver gain, 10×10^5; protein concentration, 50 μM for *traces A–C* and 70 μM for *trace D*.

by the reduced mediators. However, it can be easily identified because the resonances at $g = 2.05$ and $g = \sim 1.94$ were well separated from the radical signal. The midpoint redox potential for the development of this complex signal was determined to be −305 mV. The titration data fitted better with an $n = 2$ type redox titration curve than with an $n = 1$ type curve (Fig. 3), indicating that there must exist a strong cooperative effect for the reduction of the two [4Fe-4S] clusters. In a study of the effect of redox potential on the hydrogen production of *Megasphaera elsdenii* hydrogenase, an $n = 2$ type redox titration curve was also found for a redox potential-dependent H_2 production activity (Van Dijk and Veeger, 1981). This study suggested that in *M. elsdenii* hydrogenase, transfer of electrons from the reduced $[4Fe\text{-}4S]^{1+}$ clusters to, presumably, the hydrogen reduction site was also a cooperative process.

DISCUSSION

Evidence gathered so far indicates that the [Fe] hydrogenase from *D. vulgaris* contains, most probably, three Fe-S clusters. Two of the clusters are ferredoxin-type [4Fe-4S] clusters and are EPR-silent in the oxidized enzyme. When the enzyme is fully reduced, both clusters are in the $[4Fe\text{-}4S]^{1+}$ states and exhibit a characteristic spin-spin interacting signal. The function of these two clusters is likely to be electron transfer. Strong cooperativity between these two clusters during their reduction was suggested by our redox titration data. The third cluster is probably the hydrogen-binding site and may be responsible for the observed rhombic 2.06 and 2.10 signals. Although there exist enough spectroscopic data to suggest that this proposed H_2-binding cluster

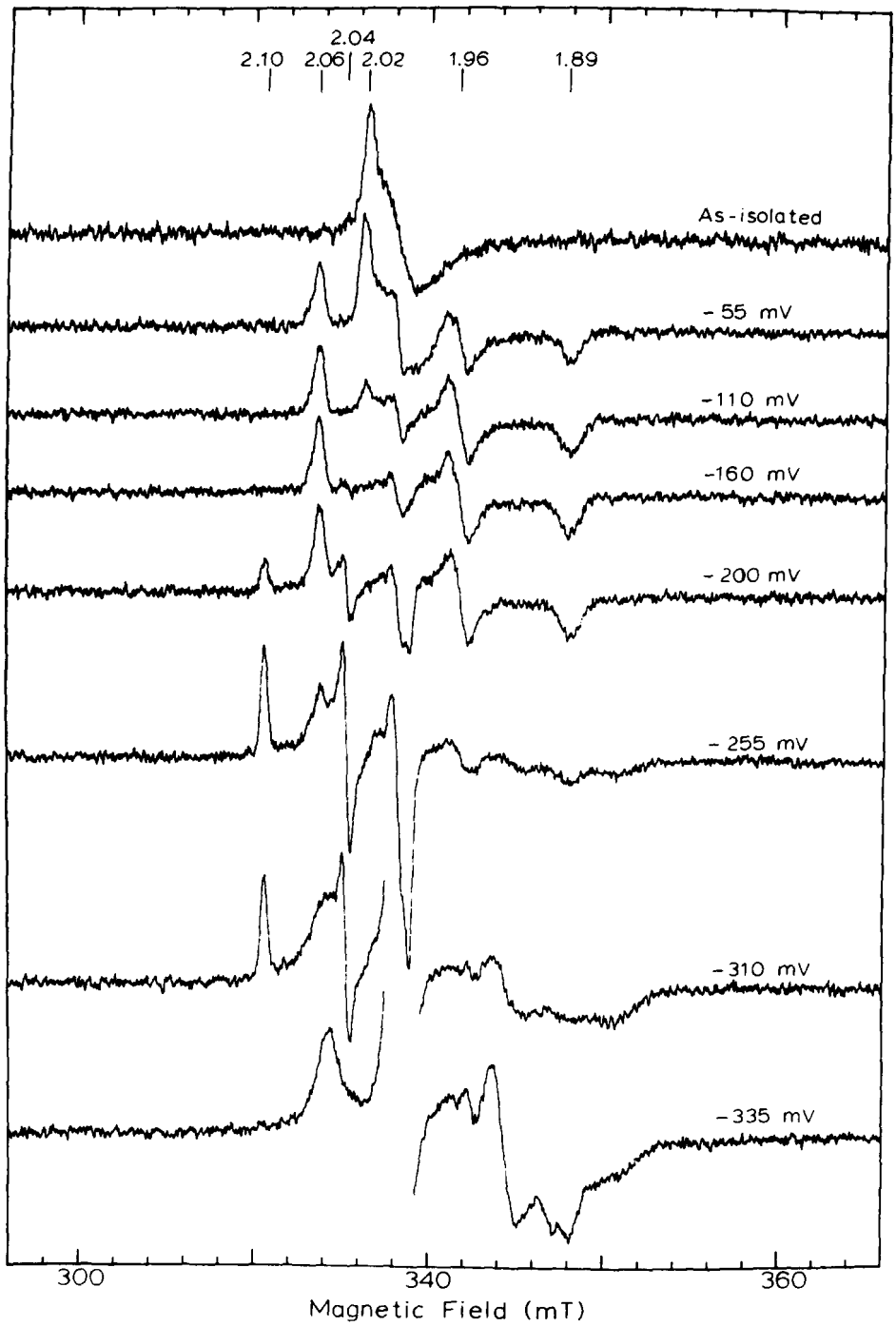

FIG. 2. **EPR spectra of *D. vulgaris* hydrogenase showing developments of EPR-active species during reductive titration.** The potentials at which the samples were poised are indicated. Other experimental conditions were: temperature, 8 K; microwave frequency, 9.43 GHz; microwave power, 2 μW; modulation amplitude, 1.0 millitesla (*mT*); receiver gain, 10×10^5; protein concentration, 50 μM.

is different from a ferredoxin-type [4Fe-4S] cluster, the composition and structure of this cluster remains undetermined.

The observation of the rhombic 2.10 signal in *D. vulgaris* hydrogenase was first reported by Hagen *et al.* (1986b) during reoxidation of the enzyme under argon atmosphere. Similar rhombic 2.10 signals were also observed for other [Fe] hydrogenases from *M. elsdenii* (Van Dijk *et al.*, 1980) and *Clostridium pasteurianum* (Erbes *et al.*, 1975). In the case of *C. pasteurianum* hydrogenase I, the rhombic 2.10 signal was assigned to an unusual iron-sulfur cluster. Mössbauer, EPR, and electron nuclear double resonance studies of the *C. pasteurianum* hydrogenase indicated that the cluster responsible for the rhombic 2.10 signal contained 3–4 iron atoms (Wang *et al.*, 1984). The magnetic hyperfine constants obtained for the iron atoms were only half of those of the iron atoms in a normal $[4Fe\text{-}4S]^{1+/3+}$ cubane cluster. This unusual cluster was proposed to be the substrate-binding site (Rusnak *et al.*, 1987; Adams, 1987), and the presence of the rhombic 2.10 signal in all three [Fe] hydrogenases strongly indicated that this cluster may be a common prosthetic group for the [Fe] hydrogenases. A recent Raman spectroscopic study on the oxidized *C. pasteurianum* hydrogenase I (Macor *et al.*, 1987) showed resonance bands characteristic of a [2Fe-2S] cluster. An H_2-binding center consisting of a [2Fe-2S] cluster magnetically coupled to a single iron was therefore suggested. Based solely on iron content determinations, a cluster containing 6 iron atoms has also been proposed for the H_2-binding site (Hagen *et al.*, 1986a). As presented in this manuscript, depending on the method used for protein determination, a range of 9–14 iron atoms/molecule can be obtained, which would suggest

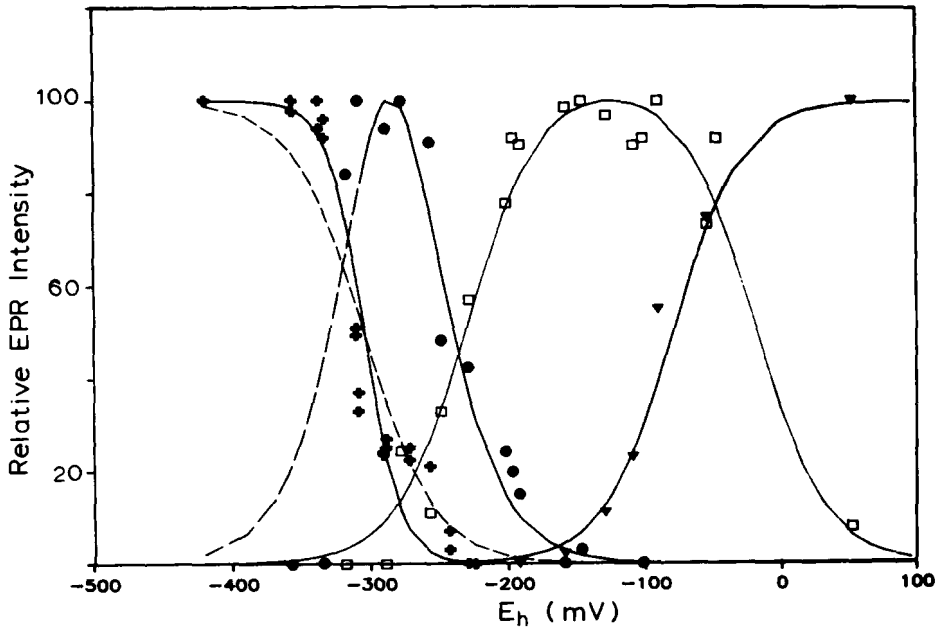

FIG. 3. **Plots of relative intensities of characteristic EPR spectra as function of applied redox potential.** ▼, intensities of the isotropic 2.02 signal; □, rhombic 2.06 signal; ●, rhombic 2.10 signal; +, complex signal of the reduced enzyme. Curves were calculated from the Nernst equation. One-electron redox processes were assumed for the isotropic 2.02 species, the rhombic 2.06 species, and the rhombic 2.10 species. The development of the complex EPR signal (+) was fitted better with a 2-electron process (——) than with a 1-electron process (- - -).

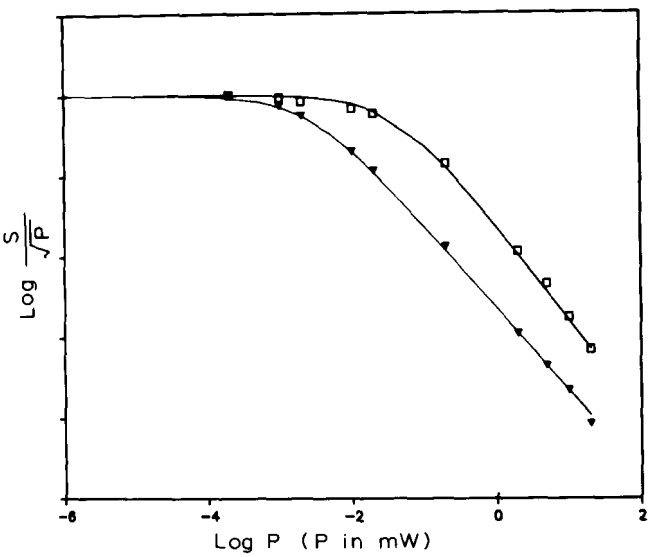

FIG. 4. **Saturation behaviors of rhombic 2.06 signal (□) and rhombic 2.10 signal (▲) measured at 8 K.** The data were fitted with an equation of the form $S \propto \sqrt{P}/(1 + P/P_{50})^{b/2}$, where S is the relative signal intensity (Rupp *et al.*, 1978). The parameters obtained were: $P_{50} = 40$ μW and $b = 1.17$ for the rhombic 2.06 signal and $P_{50} = 25$ μW and $b = 1.02$ for the rhombic 2.10 signal.

that the H_2-binding site may contain 1–6 iron atoms. (The two [4Fe-4S] clusters account for 8 of the iron atoms.) Due to the difficulties in determining protein and iron concentrations, the number of iron atoms/molecule contains a large uncertainty, and conclusions regarding iron cluster composition reached solely from protein and iron determinations must be viewed with skepticism and require support of other experimental evidence.

The rhombic 2.06 signal is a newly discovered signal for any hydrogenase.[3] The measured g-values for this rhombic 2.06 signal are quite similar to those of a reduced ferredoxin-type $[4Fe\text{-}4S]^{1+}$ cluster (Orme-Johnson and Sands, 1973). However, this signal is unusual in the sense that it appears at a redox potential that is almost 300 mV more positive than the potential at which a ferredoxin-type [4Fe-4S] cluster would be reduced. Although a combination of factors are likely to play important roles in modulating the value of midpoint redox potential, the nature of the redox center, such as the composition of the cluster's constituents or its ligands, is expected to be an essential factor in determining its redox properties. From the model compound studies of [4Fe-4S] clusters, it has been shown that substitution of thiolate by carboxylate ligands increased the midpoint potential by approximately 100 mV/substituted ligand (Johnson and Holm, 1978). The unusually high midpoint potential found for the rhombic 2.06 species therefore may suggest that it is structurally different from a ferredoxin-type [4Fe-4S] cluster. The only other iron-sulfur cluster which exhibits EPR and redox properties similar to this rhombic 2.06 species is a 4-iron cluster found in the adenylylsulfate reductase from *D. gigas* (Lampreia *et al.*, 1987). Unfortunately, the structure and ligand environment of the iron cluster in *D. gigas* adenylylsulfate reductase is also undetermined.

Another unusual character of the 2.06 signal is that it can be observed at temperatures up to 40 K with no observable broadening. The EPR signals of ferredoxin-type $[4Fe\text{-}4S]^{1+}$ clusters are generally quite broad or undetectable at 40 K. In this respect, the rhombic 2.06 signal is similar to those of reduced 2-iron ferredoxins (Orme-Johnson and Sands, 1973). Its power saturation behavior was also found to resemble that of a reduced [2Fe-2S] cluster. It is interesting to note that a resonance Raman study of the *C. pasteurianum* [Fe] hydrogenase I showed characteristic [2Fe-2S] resonances (Macor *et al.*, 1987). However, typical [2Fe-2S] spectra were not found in our preliminary Mössbauer study.

The present reductive titration study has revealed some unique and puzzling features of the species associated with the rhombic 2.06 and 2.10 signals: both the rhombic signals exhibit bell-shape titration curves. In general, a bell-shape curve represents the existence of three oxidation states which differ by 1 eq of electron. But, protein-bound iron-sulfur clusters, including [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters, were found to function only in two 1-electron inequivalent

[3] While this work was in progress, Dr. W. R. Hagen briefly reported the presence of the rhombic 2.06 signal in *D. vulgaris* hydrogenase as a minor species at the Second Workshop on Photochemical Processes for Producing Energy-rich Compounds, September 22–25, 1987, Carmona, Spain.

oxidation states. Even though the [4Fe-4S] cluster is stable in three oxidation states, namely, the $[4Fe\text{-}4S]^{1+}$, $[4Fe\text{-}4S]^{2+}$, and $[4Fe\text{-}4S]^{3+}$ states, it only operates between the 3+ and 2+ states in high potential iron-sulfur protein and between the 2+ and 1+ states in ferredoxin. Furthermore, in *C. pasteurianum* hydrogenase, the rhombic 2.10 signal shows a simple two-state curve during redox titrations (Adams, 1987). Consequently, the bell-shape curves observed for the two rhombic signals in *D. vulgaris* hydrogenase are highly unconventional. In order to retain the two-state hypothesis for the center(s) associated with the rhombic signals and considering the unlikely possibility of the existence of two unusual clusters in an enzyme, we propose that both the rhombic 2.06 and 2.10 signals probably represent the same cluster but in two different conformations.[4] The disappearance of the rhombic 2.06 signal and concomitant appearance of the rhombic 2.10 signal may not be a redox process, but probably an indication of a redox potential-dependent conformational change of the cluster. Such a conformational change can be examined by Mössbauer spectroscopy, and this line of study is now underway. The abrupt disappearance of the rhombic 2.10 signal, however, remains elusive at present.

From the above discussion, it is obvious that extensive spectroscopic investigations have been performed in an effort to characterize the iron-sulfur centers in the [Fe] hydrogenase. Characteristic and unique spectroscopic data have been obtained implicating a novel structure for the H_2-binding site. However, it is equally obvious that there exist many interesting problems concerning the structure of the proposed H_2-binding center which remain to be resolved.

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[4] For example, the rhombic 2.10 signal may represent a hydrogen-bound cluster, and the rhombic 2.06 signal an unligated cluster.