Electron paramagnetic resonance and other properties of hydrogenases isolated from *Desulfovibrio vulgaris* (strain Hildenborough) and *Megasphaera elsdenii*

Hans J. GRANDE, William R. DUNHAM, Bruce AVERILL, Cees VAN DIJK, and Richard H. SANDS

Department of Biochemistry, Agricultural University, Wageningen;
Biophysics Research Division, University of Michigan, Ann Arbor, Michigan; and
Department of Chemistry, University of Virginia, Charlottesville, Virginia

The hydrogenases of *Desulfovibrio vulgaris* and *Megasphaera elsdenii* are compared with respect to some of their physical properties. In addition to Fe the only metal ions that are present in significant amounts are Ni and Cu. From cluster extrusion experiments it follows that the *D. vulgaris* enzyme contains three 4 Fe-4 S clusters, while *M. elsdenii* hydrogenase only releases part of its Fe-S clusters. The resting *D. vulgaris* enzyme shows only a small 3 Fe-xS type of EPR signal (maximum 5 % electron equivalent). This amount can be increased to approximately 25 % by treatment with ferricyanide, with a concomitant large decrease in activity. The *M. elsdenii* enzyme shows in its oxidized state a normal Hipip (high-potential iron-sulphur protein) type of EPR spectrum. After a reduction/oxidation cycle the *D. vulgaris* enzyme also shows a weak Hipip type of EPR spectrum. In the reduced state both enzymes show complex spectra. By integration of those spectra it is shown that 1.5 electron equivalents are present. The complex spectra do not arise from nuclear hyperfine interactions but are partially due to electron spin interactions. It is proposed that the spectrum of reduced *D. vulgaris* hydrogenase consists of a sum of three different ferredoxin-like spectra.

Although hydrogenases are well-studied enzymes, little is known about the way in which they are internally organized. Moreover, the identities of the components of the active site are unknown, as are the features that make some hydrogenases 100 times more active than others.

Apparently some hydrogenases function mainly as H_2-production enzymes, while others favor the H_2 uptake. In a recent review [1] Adams et al. compared a large number of hydrogenases originating from different sources. Their main conclusion relevant to this article was the observation that all hydrogenases have at least a so-called Hipip (high-potential) 4Fe-4S cluster in common, as indicated by an EPR signal in the oxidized state, with g values with an average above 2 [1]. They suggested, therefore, that this Hipip site might be the active site. They also compared EPR properties of those enzymes for which EPR data are available.

In this article we report results from two highly active enzymes, from *Desulfovibrio vulgaris* and *Megasphaera elsdenii*, which have been extensively examined by EPR-spectrometric and kinetics methods [2—5]. These enzymes, in particular the one from *M. elsdenii*, resemble the hydrogenase isolated from *Clostridium pasteurianum*. The published EPR spectra of *C. pasteurianum* hydrogenase are very similar to our spectra [6]. It is by now established for *C. pasteurianum* that it contains, just as the hydrogenases of *D. vulgaris* and *M. elsedenii*, 12 Fe and

about 12 S [7], or three 4Fe-4S clusters, as follows from extrusion experiments [8]. From EPR spectral studies it has been determined that both *M. elsdenii* and *C. pasteurianum* contain at least one Hipip type of cluster. A more extensive study, especially with respect to EPR, seems to be appropriate to analyze the (dis)similarities between the hydrogenases of *D. vulgaris* and *M. elsdenii*. Although both are highly active they differ in several properties. Extensive kinetic studies on these enzymes showed some significant differences: most importantly the difference in redox behaviour and salt inhibition instead of stimulation for *D. vulgaris* versus *M. elsdenii* hydrogenase [2,5]. The EPR spectra of the enzymes also differ and could help to test the mechanism proposed by us [9]. In this study we present evidence for the existence of three 4Fe-4S clusters in each enzyme, analyze their nature, as well as provide evidence for the nature of the active site.

MATERIALS AND METHODS

Materials

Desulfovibrio vulgaris (strain Hildenborough, NCIB 8303) was cultivated at 35 °C in the modified Saunders medium [10], pH 7.2 and 50 μM EDTA [11] stirred under N_2 gas in 20-l glass bottles. The hydrogenase was extracted and purified according to Van der Westen et al. [12]. *Megasphaera elsdenii* (strain LC1 of Elsden et al.[13]) was grown on lactate/yeast extract according to the method described by Walker [14] in iron-rich medium. The hydrogenase was purified according to Van Dijk et al. [2].

Spinach ferredoxin and *Clostridium pasteurianum* ferredoxin were obtained from Sigma Chemical Co. Hexamethyl-phosphoramide (HMPA) was obtained from Aldrich

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Abbreviations. HMPA, hexamethylphosphoramide; o-xyl(SH)₂, α,α'-dimercapto-o-xylene; cyt c_3, cytochrome c_3 from *D. vulgaris* (Hildenborough); EPR, electron paramagnetic resonance; PIXE spectroscopy, proton-induced X-ray emission spectroscopy; Hipip, high-potential iron-sulphur protein.

Enzymes. Hydrogenase or hydrogen: cytochrome c_3 oxidoreductase (EC 1.12.2.1); hydrogenase or hydrogen: ferredoxin oxidoreductase (EC 1.18.3.1).

Chemical Co., purified by fractional distillation from calcium hydride *in vacuo* and stored under nitrogen. α,α-Dimercapto-o-xylene [o-xyl(SH)₂] was prepared by a published method [15] while thiophenol (Aldrich) was purified by vacuum distillation before use.

Methods

The techniques and apparatus used for displacement (extrusion) of Fe-S clusters from proteins have been described previously [8,16−18]. Hydrogenases were examined using both a direct displacement method [8,16] with thiophenol, and a double displacement reaction [16,18] in which the Fe-S centers are initially removed with o-xyl(SH)₂, with sequential ligand substitution by thiophenol. In both cases quantification was by spectrophotometric methods [8,16,17] using a Cary 17 spectrophotometer. Samples of hydrogenase (50−70 μM) in 0.21 ml 250 mM Tris/HCl buffer, pH 8.0, were treated at room temperature with 0.8 ml 24 mM thiophenol or 12 mM o-xyl(SH)₂ in HMPA. The reactions were monitored spectrophotometrically in the double-septum-seal cuvettes described previously [16], until the reaction reached completion (< 10 min). For *D. vulgaris* hydrogenase control reactions with spinach ferredoxin and *C. pasteurianum* ferredoxin were performed as well; absorption coefficients, determined in these control experiments, agreed well with those reported previously [8,16,17].

Displacement of water by ²H₂O was accomplished by dialyzing twice against a tenfold excess of deuterated buffer solution. The Hepes (pH 8.0) used was not deuterated since a relative low concentration was used (50 mM). Deuteration of the enzyme solutions was at least 90%. Reduction of both hydrogenases was performed at two pH values (6.5 and 8.0) and was accomplished either by dithionite treatment or by placing a sample for 45 min under deuterium gas. Oxidation, in the case of *M. elsdenii* hydrogenase, was done as described earlier [3], by shifting the pH with phosphate buffer to 6.0 and adding sulfite. EPR measurements at X band were performed on a Bruker 200D spectrometer, to which a DATA General NOVA-3 computer was attached, or on a Varian-E-12. Integrations were done numerically and compared with a copper perchlorate standard (pH 2). The appropriate correction for a field-swept spectrum $(\delta B/\delta V)$ [19], temperature etc. were applied. Spectra at other frequencies (15.0 GHz (P band) and 3.0 GHz (S band) were performed on home-built instruments [20−22]. The instruments used can all accommodate normal X-band tubes. The temperature was measured just below the sample by the resistance of Allen Bradley carbon resistors. On the Bruker EPR spectrometer a home-built He accessory was used [23] while on the other instruments a Helitran He-flow type of cryostat was used. Fields were calibrated either with an AEG nuclear resonance magnetic-field meter (on the Bruker spectrometer) or with a digital NMR gaussmeter, Systron Donner, model 3193. Frequency was measured on each sample with either Hewlett Packard frequency meters HP 536A (S band), HP 532B (X band), HP P532A (P band) or with a Systron Donner Counter 1017 model 129A on the Bruker spectrometer. Metal quantification was performed on two identical samples for each protein. Metal composition was measured with proton-induced X-ray emission (PIXE) spectroscopy [24] at Eindhoven University, Department of Physics. 50 μl protein solution was brought onto a small support. The sample was thereafter allowed to dry at room temperature. The beam is adjusted to the sample size.

Since a large Zn background was found, the presence of Zn in the hydrogenase cannot be excluded. For nickel, atomic absorption was also used. A calibration series of Ni in bovine serum albumin was used to calculate the concentrations from atomic absorption data.

RESULTS AND DISCUSSION

CLUSTER EXTRUSION EXPERIMENTS

Our previous work has shown that the enzymes from both *Megasphaera elsdenii* [2] and *Desulfovibrio vulgaris* [12] contain 12 ± 1 iron and 11 ± 2 sulfur atoms/molecule of enzyme. It is, therefore, acceptable to assume that both enzymes contain three of the 4Fe-4S type of clusters, although a report of a hydrogenase with 2Fe-2S clusters has been published [25]. Cluster extrusion, if successful, is a technique which is highly indicative of the kind and number of clusters present. Treatment of an iron-sulfur protein in denaturing organic solvent with excess thiol results in displacement of the (presumed) cysteinyl mercaptide ligands of the protein and release of the Fe-S clusters with simple mercaptide ligands, $[Fe_4S_4(SR)_4]^{2-}$ and/or $[Fe_2S_2(SR)_4]^{2-}$. The latter can be identified and quantified by comparison of their spectroscopic properties with those of the known synthetic complexes; in the absence of interference from other chromophores, electronic absorption spectroscopy is the method of choice [8,16, 17].

In Fig. 1 the optical spectrum of *D. vulgaris* hydrogenase, treated sequentially with o-xyl(SH)₂ and thiophenol in 80% v/v HMPA, is compared with the spectrum of *Clostridium pasteurianum* ferredoxin and spinach ferredoxin obtained under the same conditions and at approximately the same total iron concentration. The position of λ_{max} (458 nm) and the overall features of the hydrogenase spectrum show clearly that the hydrogenase contains 4Fe-4S cores [17]. This is substantiated by the optical spectrum of hydrogenase treated only with o-xyl(SH)₂ in 80% v/v HMPA (not shown), which was very similar in both overall shape and peak positions to that obtained with *C. pasteurianum* ferredoxin, while bearing no similarity to that of spinach ferredoxin [16]. Use of known absorption coefficients [8,16,17] gave 3.0 and 2.8 4Fe-4S clusters/molecule, based on the o-xyl(SH)₂ and o-xyl(SH)₂ plus thiophenol data respectively. An analogous experiment using thiophenol alone gave a spectrum identical to that shown in Fig. 1; quantification gave a value of 2.9 4Fe-4S clusters. Even at a relatively low thiophenol concentration (19 mM) no evidence was found for spectral features at 515 nm and 580 nm. These features were observed under comparable conditions [17] for *Azotobacter vinelandii* iron-sulfur protein III, the first example of a protein shown to contain a trinuclear iron-sulfur cluster [26,27], and have been tentatively ascribed to the presence of this unusual Fe-S prosthetic group. Even with the interference from the tetranuclear cluster, distinct long-wavelength shoulders were observed in that case at 515 nm and 580 nm during displacement experiments [17]. Upon prolonged standing or treatment with a large excess of thiophenol (100 mM) these shoulders disappeared and the resulting spectrum resembled that of a mixture of 4F-4S and 2Fe-2S clusters. With o-xyl(SH)₂ as complexing agent no unusual intermediate spectral features were observed for *A. vinelandii* iron-sulfur protein III; only a spectrum again consistent with a mixture of dimeric and tetrameric clusters. The absence of similar features in the spectrum of hydrogenase under conditions specifically de-
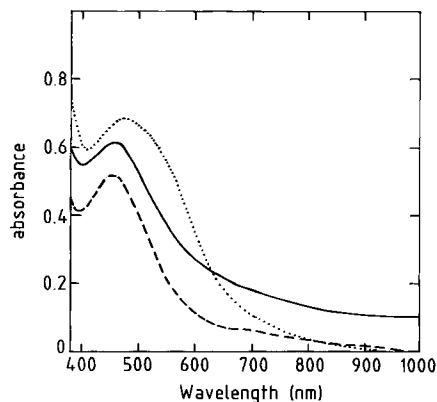
Fig. 1. *Optical spectrum of HMPA-extruded* D. vulgaris *hydrogenase.* Proteins are in 80% v/v HMPA containing 50 mM Tris/HCl, pH 8.0, 0.6 mM o-xyl(SH)$_2$, and 60 mM thiophenol prepared as described in Materials and Methods. The total iron concentration is 115 μM in each case; the optical path length is 1.0 cm ($\cdots$) Spinach ferredoxin (57.5 μM); (———) *D. vulgaris* hydrogenase (10.0 μM), offset by +0.1 A; (———) *C. pasteurianum* ferredoxin (14.4 μM)

signed to optimize the chances of the observation of the trinuclear iron sulfur cluster (low thiophenol) and use of HMPA, which typically causes slow unfolding of proteins [17], suggests that no such unit is present in appreciable amounts. A definite conclusion on this point will require a careful Mössbauer spectroscopic study, which will be undertaken.

The results of the cluster displacement (core extrusion) experiments indicate that each *D. vulgaris* hydrogenase molecule contains three 4Fe-4S clusters. Uncertainties in peak positions and line shapes are such that it is not possible to state categorically that no 2Fe-2S clusters are present. With 12 Fe and 12 S per molecule, however, the presence of a stoichiometrically significant amount of 2Fe-2S center would require two 2Fe-2S and two 4Fe-4S clusters, which is not in agreement with our observations. Furthermore, no evidence for the presence of a stoichiometrically significant amount of trinuclear iron-sulfur center was found.

The above conclusion, regarding the identity and number of iron-sulfur prosthetic groups, is consistent with available data on similar enzymes from other organisms. Thus, based on cluster displacement experiments, preparations of hydrogenase from *C. pasteurianum* are reported to contain either one [28] or three 4Fe-4S units [8], while hydrogenase from an organism closely related to that examined here, *Desulfovibrio gigas*, has been shown to contain three 4Fe-4S clusters per molecule [29]. Similar experiments on hydrogenase from a different strain (Miyazaki) of *D. vulgaris* suggested the presence of two 4Fe-4S centers [30,31]. To date, there is no published evidence from cluster displacement (core extrusion) studies for the presence of anything but tetranuclear Fe-S centers in any hydrogenase. Recently, however, solvent-perturbed EPR studies on the unusual FMN-containing soluble hydrogenase of *Alcaligenes eutrophus* led to the conclusion that there were two 2Fe-2S and two 4Fe-4S centers per molecule [25]. Apparently tetranuclear iron-sulfur centers are necessary cofactors for the hydrogenase reaction. A mechanism based on the unique ability of 4Fe-4S centers to act as two-electron oxidants or reductants has been proposed [32].

Displacement of the iron-sulfur clusters from *M. elsdenii* hydrogenase was also attempted. In this case, however, the iron-sulfur clusters were not readily released from the protein
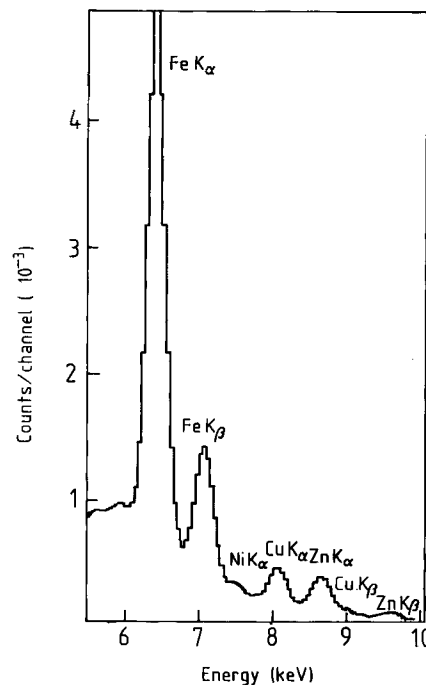

Fig. 2. *Proton-induced X-ray emission spectrum of* D. vulgaris *hydrogenase.* The vertical axis reflects the number of counts per channel of the multichannel analyzer. The intensity is normalized to the Fe K$_\alpha$ peak. The emission lines are indicated in the figure

under conditions similar to those used for the *D. vulgaris* enzyme [80% HMPA, pH 8.0, 24 mM PhSH or 12 mM o-xyl(SH)$_2$], as indicated by only minor spectral changes over a period of seven days at room temperature. This suggests that the enzyme is more resistant to denaturation by organic solvents; conditions under which the iron-sulfur clusters can be removed without precipitation of the protein or apoprotein have not yet been found.

OTHER METALS

It has recently been reported that, in addition to Fe, other metals, most importantly Ni, are present in hydrogenases [33 – 37]. In order to look for this metal in our enzymes a more general method was used. Protons accelerated in a cyclotron to an energy of 3.5 MeV were allowed to strike a protein sample (see Materials and Methods). Those protons induce an X-ray emission whose energy is characteristic for each metal and from whose intensity the amount can be calculated. In Fig. 2 the low-energy part of the PIXE spectrum of *D. vulgaris* hydrogenase is shown. The first peaks are the Fe K_α and Fe K$_\beta$ lines and represent clearly the largest amount of metal present. The characteristic lines for Cu and Zn are also clearly observed. A very tiny peak between the Fe K$_\beta$ line and the Cu K$_\alpha$ line can be ascribed to nickel. Atomic absorption experiments on protein of the same batch gave an amount of 0.7 ± 0.1 Ni atoms/molecule of protein. If this number is used to calculate the intensity of the nickel K$_\alpha$ in the PIXE spectrum, a three-times bigger peak in the spectrum would have been expected. In the case of *M. elsdenii* enzyme (not shown) somewhat more Ni and less Cu is observed in the PIXE spectrum. In atomic absorption experiments on this enzyme, different amounts of Ni were found (0.4 – 0.8 Ni

atoms/molecule). Since Ni is close in atomic number to Fe an accurate value is difficult to obtain from PIXE without suitable resolution enhancement. By comparison with the Cu K_α peak (0.2 Cu atom/molecule) a value of 0.4 ± 0.2 Ni/ molecule can be estimated in the case of *M. elsdenii* hydrogenase.

No significant amounts (> 1 ppm weight fraction) of elements other than Cu, Zn and, in the high-energy range, Br are detected within the energy range studied. Cu was found in significant amounts but in both *D. vulgaris* (0.5 Cu atom/ molecule maximal, but in other preparations less) and *M. elsdenii* hydrogenase less than stoichiometric. Since some Ni is found, EPR spectra also were run of several samples in the region of interest. No EPR signal at the published g values of 2.3, 2.2 and 2.0, ascribed to Ni [33], was detected at 85 K or 10 K in any oxidized or reduced sample of either enzyme. Since Mössbauer and MCD spectra of *D. vulgaris* hydrogenase (unpublished results) show that in the oxidized state (as isolated) Fe is mainly diagmagnetic, the Ni must also be diamagnetic or strongly coupled to a cluster such that an $S = 0$ state results, or be in an EPR-undetectable paramagnetic state. These results suggest that the Ni is in this case clearly different from those reported elsewhere [33−37]. A function of Ni in catalysis by hydrogenase has yet to be proven. Hydrogenase is a negatively charged protein as judged from its affinity for DEAE-cellulose and is, therefore, expected to bind metals readily. In any case a link with activity must be proven before a function can be ascribed to a particular metal. In our view the Cu and possibly also the Ni, observed in our preparations, are most probably adventitious contaminants.

EPR RESULTS

Some EPR results on the *M. elsdenii* enzyme have been reported [3], while recently some relevant EPR spectra of *D. vulgaris* hydrogenase have been published [4].

Oxidized enzymes

The spectrum of *D. vulgaris* hydrogenase, as isolated in Fig. 3 A, is what can be called the 'oxidized' enzyme spectrum. From the above results it follows that there are only 4Fe-4S clusters. This spectrum, however, is very similar to that of an oxidized 3Fe-xS cluster [38], characterized by very-close-lying g values of the main axes centered around 2.02 and a particular broad line width in the g region. Integration of the signal, however, results in only about 5% of one electron equivalent. Furthermore, no Hipip type of spectrum is observed under these conditions (as isolated). A Hipip type of spectrum is characterized by g values on the average above 2. Characteristic g values are 2.115 and 2.087 (axial) for Hipip itself and for hydrogenases 2.1, 2.05 and 2.00. The spectrum of high-potential iron protein arises from a 4Fe-4S cluster in its oxidized state $(4Fe-4S)^{3+}$. The *M. elsdenii* enzyme exhibits a Hipip type of signal with an intensity of 0.6 spin at a redox potential of about -0.25 V [3]. Other oxidized hydrogenases also have just such a signal as Adams et al. [1] pointed out in their review. Although their interpretation of 'Hipip-like' is questionable in view of the current knowledge on 3Fe-xS clusters. Next to *M. elsdenii* only *C. pasteurianum* hydrogenase is clearly of this type; many other spectra (see e.g. [36]) might be of the oxidized 3Fe-xS type. Since *D. vulgaris* hydrogenase is also of the very active type it was expected that one of the three 4Fe-4S clusters would be of the Hipip type, and an EPR signal with g values around
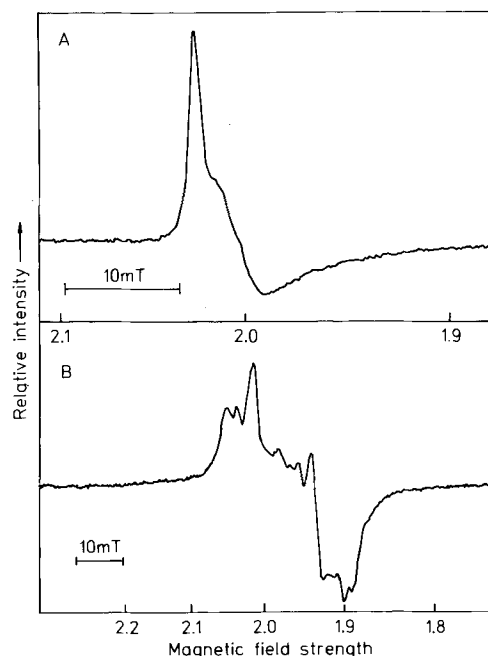

Fig. 3. *Electron spin resonance spectrum of oxidized and reduced* D. vulgaris *hydrogenase.* (A) 228 μM protein, as isolated in 50 mM Hepes pH 8.0. EPR conditions: frequency 9.428 GHz, modulation amplitude 0.5 mT, field sweep 319.1−358.8 mT, gain 1.6×10^5, power 2 mW, temperature 9.1 K. (B) 100 μM protein, pH 8.0 in 50 mM Hepes, after 45 min at 1 atm (101 kPa) hydrogen. EPR conditions: frequency 9.417 GHz, modulation amplitude 1 mT, field sweep 286.8−386.0 mT, gain 3.2×10^5, power 0.51 mW, temperature 10.8 K. The labeling of the x axis is in absolute g values

2.1, 2.05 and 2.0 was expected under aerobic conditions. Treatment of the *D. vulgaris* enzyme with a tenfold excess (approx. 1 mM final concentration) ferricyanide results in an increase of the EPR signal to an intensity which corresponds to about 0.25 spin, without an observable change in line shape of the 3 Fe-xS signal. The enzyme thus treated has about 50% of its original hydrogenase activity. Further addition, up to a 100-fold excess, does not increase the signal any more, but rather decreases it. At a 100-fold excess the hydrogenase activity is only 4% of the original activity. Oxidation with another strong oxidizing agent, WCl_6, did not give any increase of the 3Fe-xS type of spectrum, while the activity also declined to almost zero.

The absence of a Hipip type of signal in the oxidized state and the extrusion of three 4Fe-4S clusters indicate that in *D. vulgaris* hydrogenase only $(4Fe-4S)^{2+,1+}$ clusters are present, which can assume the 2+ or 1+ oxidation level. Moreover, Mössbauer and MCD spectra (unpublished results) show only a minor amount of paramagnetic species in this state (as isolated). The *M. elsdenii* enzyme is isolated under anaerobic conditions in the presence of dithionite [2]. If dithionite is removed and the electrons transferred to flavo-doxin a ($g = 2.09$, 2.04 and 2.00) spectrum results which is slightly different from that published by us [3]. The spectrum in [3] is obtained by shifting the pH and adding sulfite, resulting in a final potential of about -0.25 V. A similar spectrum is also obtained for *C. pasteurianum* hydrogenase [6] oxidized with ferricyanide. Addition of ferricyanide is in that case spectroscopically reversible [39]. It is, therefore, clear that *M. elsdenii* hydrogenase is more similar to *C. pasteurianum* hydrogenase while the *D. vulgaris* enzyme differs.

Reduced enzymes

The spectrum of reduced *D. vulgaris* enzyme is shown in Fig. 3 B. This spectrum is very similar to the spectra published by us for *M. elsdenii* [3] and for the *C. pasteurianum* enzyme [6]. This spectrum can be obtained either by reduction with dithionite or by incubation under 1 atm (101 kPa) hydrogen. The intensity of the spectrum is when integrated equivalent to about 1.5 electrons, based on a copper standard. A similar amount is found for reduced *M. elsdenii* hydrogenase. Mortenson [39] suggested that for *C. pasteurianum* hydrogenase this type of spectrum might result from spin-spin interaction between the clusters as has been shown previously for the *Megasphaera lactilyticus* ferredoxin by Mathews et al. [40]. Another cause could be a strong hyperfine coupling. In any case it is clear that the spectra observed for reduced *M. elsdenii*, *D. vulgaris* and *C. pasteurianum* hydrogenases cannot easily be explained by two non-interacting (4Fe-4S)$^{1+}$ clusters. In the following section we examine whether this spectrum can be explained in terms of either spin-spin or hyperfine interaction.

The problem to be solved is how large the hyperfine term and the spin-spin interaction terms are relative to the Zeeman energy in the Hamiltonian. When only two spins are present the Hamiltonian is:

$$H = \beta \vec{S_1} \cdot \tilde{g}_1 \cdot \vec{B} + \beta \vec{S_2} \cdot \tilde{g}_2 \cdot \vec{B} + \vec{S_1} \cdot \tilde{D} \cdot \vec{S_2} + \vec{I_1} \cdot \tilde{A} \cdot \vec{S_1}$$
$$+ \vec{I_2} \cdot \tilde{A}_2 \cdot \vec{S_2}.$$

In this equation the tensor D includes both the isotropic exchange coupling J as the dipolar coupling. From this Hamiltonian it is clear that only the Zeeman terms will be magnetic-field-dependent. A question arises about the origin of I_1 and I_2. Since both the naturally occurring Fe and S nuclei do not contribute to the hyperfine interaction ($I = 0$), this would have to come from a different source. The nuclei, ^{1}H and ^{14}N, are present in large numbers in amino acids that are likely to be ligands to a 4Fe-4S cluster. Several studies [41,42] on ferredoxins have shown that those couplings are of some importance and are of the order of a few gauss [42].

Experiments in which the buffer was exchanged for deuterated buffer did not show any evidence of a change in the line width of any of the species studied. The spectra of oxidized hydrogenases have relative narrow line widths and do not show any narrowing. Therefore, it is clear that co-ordinated dihydrogen (dideuterium) does not interact with these EPR-visible oxidized species. In the case of partially reduced enzyme both *M. elsdenii* and *D. vulgaris* hydrogenase have very complex EPR spectra, which are due to overlap of at least two species. Since we expect many of the interacting nuclei to be non-exchangeable, as they were in the algal ferredoxin studies [41], it is expected that the visual effect of the magnetic hyperfine coupling from exchangeable protons is small. Since no effect is observed, apparently no direct interaction with dihydrogen is present. Moreover, the hyperfine interaction is therefore clearly not the cause of the complexity of the spectrum. This leaves the spin-spin interaction as the only possible candidate for the complexity of the spectra, if only two species are involved in case of the reduced spectrum of Fig. 3 B. As mentioned already this term is independent of the magnetic field $\vec{B}$. Changing the frequency of the experiment will cause resonance to occur at another value of $\vec{B}$. If the same sample is studied at different frequencies and the position of the absorptions is plotted on the same g scale, it is possible to distinguish genuine g values from spin-spin inter-
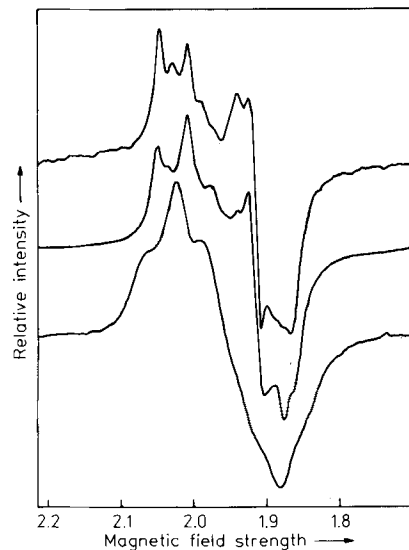

Fig. 4. *Electron spin resonance spectra of reduced* D. vulgaris *hydrogenase at three frequencies*. Enzyme (228 μM) is reduced with 15 mM dithionite at pH 8.0 in 50 mM Hepes buffer. Upper spectrum: frequency 14.765 GHz, modulation amplitude 1 mT, field sweep 432.6 – 638.4 mT, power 3 mW, temperature 19 K. Middle spectrum: frequency 9.193 GHz, modulation amplitude 1 mT, field sweep 290.1 – 391.1 mT, power 10 μW, temperature 11 K. Bottom spectrum: frequency 3.055 GHz, modulation amplitude 0.53 mT, field sweep 98.8 – 123.3 mT, power 8 mW, temperature 12 K. The labeling of the x axis is in absolute g values
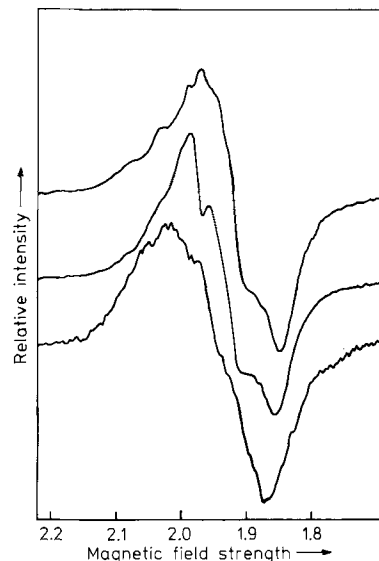

Fig. 5. *Electron spin resonance spectra of reduced* M. elsdenii *hydrogenase at three different frequencies*. The enzyme (100 μM) is in 5 mM dithionite at pH 8.0 in 10 mM Hepes buffer. Upper spectrum: frequency 14.765 GHz, modulation amplitude 1 mT, field sweep 432.2 – 638.2 mT, power 3 mW, temperature 17 K. Middle spectrum: frequency 9.198 GHz, modulation amplitude 1 mT, field sweep 290.1 – 391.1 mT, power 10 μW, temperature 11 K. Bottom spectrum: frequency 3.055 GHz, modulation amplitude 0.53 mT, field sweep 98.8 – 123.2 mT, power 8 mW, temperature 10 K. The labeling of the x axis is in absolute g values

actions (and hyperfine interaction). If the absorption is due to true g values it will occur at the same g regardless of frequency while either spin-spin or hyperfine interactions will cause an apparent shift in g as a function of frequency.

In Fig. 4 the spectra of reduced *D. vulgaris* hydrogenase and in Fig. 5 the spectra of *M. elsdenii* enzyme are plotted at three
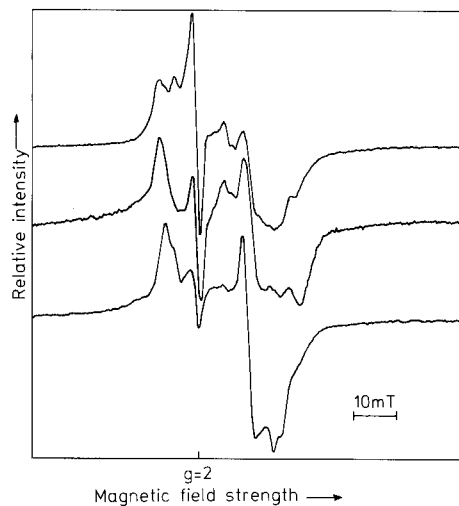
Fig. 6. *Electron spin resonance spectra of partially reduced* D. vulgaris *hydrogenase*. Enzyme concentration is 100 μM in all cases. The enzyme solutions were incubated for 45 min under hydrogen in the presence or absence of cyt c_3 (0.02 ml 4.6 mg/ml cyt c_3 was added to 0.25 ml sample). Upper spectrum: 0.32 atm (32.4 kPa) H_2, pH 6.0 (Mes 50 mM), EPR conditions: frequency 9.212 GHz, gain 1×10^4, temperature 14.5 K. Middle spectrum: 0.1 atm (10 kPa) H_2, pH 6.0 (Mes 50 mM), +cyt c_3; EPR conditions: frequency 9.210 GHz, gain 2×10^4, temperature 13.5 K. Lower spectrum: 0.1 atm (10 kPa) H_2, pH 8.0, (Hepes 50 mM) +cyt c_3. EPR conditions: frequency 9.208, gain 1×10^4, temperature 15 K. In all spectra the center field was at 340.0 mT, the sweep width 100 mT, the power 0.5 mW and the modulation 1 mT

frequencies. The frequencies are about 3.0 GHz (S band), 9.2 GHz (X band) and 15.0 GHz (P band).

Fig. 4 and 5 both show that there are some changes in appearance of the spectra of both reduced enzymes at the frequencies shown. There are some shifts and intensity differences in the middle portion of the spectra, although the overall width is about the same, at least for X and P band. At S band the features do change and the spectrum has clearly broadened. This change is partly due to magnetic hyperfine interaction, which is more easily observed at low frequency, and partly due to spin-spin interactions. This spin-spin interaction also gives rise to intensity differences and shifts that can be more easily seen in low-frequency spectra, than in higher-frequency spectra. The middle portion of the spectra shows most clearly the presence of interaction.

From the above spectra one can not determine whether the EPR features are due to two or three $(4Fe-4S)^{1+}$ clusters. The intensity of the signal of *D. vulgaris* or *M. elsdenii* hydrogenase, reduced with either dithionite or 1 atm (101 kPa) H_2, is about 1.5 electron equivalents. However, the fact that it has been shown by steady-state kinetics that both enzymes have midpoint potentials which are identical to that of the H_2/H^+ electrode [9] (for *D. vulgaris* this is true from pH 9 to pH 6 [5]) means that the enzyme is only half reduced, since it is exactly at the midpoint potential under 1 atm (101 kPa) H_2. Because of the high activity of the enzyme, dithionite reduction leads to H_2 production again stopping at the H_2/H^+ potential. The value of 1.5 electron equivalents, therefore, suggests three clusters. Fig. 6 shows spectra of *D. vulgaris* hydrogenase obtained at low hydrogen pressures. According to the above-mentioned properties this enzyme will be less reduced at $pH_2 = 0.1$ atm (10 kPa) which is indeed what is observed in the experiments represented in Fig. 6. The fea-

tures of the spectrum at low field and high field show three possible g_z and g_x values, suggesting the presence of three clusters. The free-radical signal in the middle of the spectrum is probably due to a very small amount of methyl viologen, which was present in some of the samples due to an inadequate liquid trap in the argon gas line, which allowed some of the scrubbing solution into the sample. It has already been stated in this article that the *D. vulgaris* enzyme has three different types of ferredoxin-like $[(4Fe-4S)^{2+,1+}]$ oxidation states, probably with different redox potentials.

It is also concluded that no Hipip-like cluster is present. Reduction of *D. vulgaris* hydrogenase artificially oxidized with ferricyanide revealed another property of at least one of the clusters. The oxidized enzyme could only partially be reduced with hydrogen. Reduction with dithionite resulted in a spectrum very similar but not identical to a normal enzyme EPR spectrum, which still represents, as judged by integration, 1.1–1.5 electron equivalents/molecule protein. Probably, two ferredoxin-type ($g = 2.05, 1.94, 1.86$) clusters are still intact while a third one is damaged. This is in contrast with experiments with *C. pasteurianum* [39] where the ferricyanide oxidation was reversible. Therefore one of the clusters in *D. vulgaris* is different from those in *M. elsdenii* and *C. pasteurianum*.

The special cluster in *D. vulgaris* can be reduced and gives a ferredoxin-like spectrum (Fig. 6 shows three species present). Since the EPR spectrum of reduced *M. elsdenii* enzyme (Fig. 5) is featureless, it is hard to distinguish between two or three EPR-active species present. Clearly the changes with frequency are larger and spin-spin interaction might be more important. Since it is hard to prepare a dithionite-free undamaged protein, similar experiments with hydrogen, as undertaken with the *D. vulgaris* enzyme, have not yet been performed.

A careful redox titration with hydrogen of both hydrogenases (10 atm, 0.1 atm; 1 MPa, 10 kPa) can elucidate the existence of the different species. In principle this kind of titration is best monitored by EPR spectra at different frequencies and also by Mössbauer spectra. Both types of study are currently underway and will be published by us in the near future.

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H. J. Grande and Cees van Dijk, Laboratorium voor Biochemie, Landbouwhogeschool, De Dreijen 11, NL-6703-BC Wageningen, The Netherlands

W. R. Dunham and H. Sands, Biophysics Research Division, University of Michigan, 2200 Bonisteel Boulevard, Ann Arbor, Michigan, USA 48109

B. Averill, Department of Chemistry, University of Virginia, McCormick Road, Charlottesville, Virginia, USA 22901