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# Changes in the electronic structure around Ni in oxidized and reduced selenium-containing hydrogenases from *Methanococcus voltae*

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The selenium-containing F420-reducing hydrogenase from *Methanococcus voltae* was anaerobically purified to a specific hydrogen-uptake activity of 350 U/mg protein as determined with the natural electron acceptor. The concentrated enzyme was used for EPR-spectroscopic investigations. As isolated, the enzyme showed an EPR spectrum with $g_{xyz}$ values of 2.21, 2.15 and 2.01. Illumination of such samples at low temperatures led to an EPR spectrum with $g_{xyz}$ values of 2.05, 2.11 and 2.29. These spectra are typical for [NiFe]hydrogenases in the active state. Spectra of samples enriched in $^{77}$Se showed a hyperfine interaction between the unpaired spin of the nickel ion and the nuclear spin of one $^{77}$Se atom before and after illumination. A 90° flip of the electronic z-axis is proposed to explain the hyperfine interaction in both states. This has been demonstrated previously only for the F420-non-reducing hydrogenase from *M. voltae*, where the selenium atom is present as a selenocysteine residue on an unusually small seperate subunit [Sorgenfrei, O., Klein, A. & Albracht, S. P. J. (1993) *FEBS Lett. 332,* 291–297]. The results demonstrate that the three-dimensional structures of the active sites in the selenium-containing F420-reducing and F420-non-reducing hydrogenases from *M. voltae* are highly similar and hence are not influenced by the unusual subunit structure of the latter enzyme. Oxidized samples containing either natural selenium or $^{77}$Se were prepared from the F420-reducing and the selenium-containing F420-non-reducing hydrogenase. Both enzymes exhibited EPR spectra typical for [NiFe]hydrogenases in the inactive 'ready' state. In contrast to the reduced form, no splitting of the nickel-derived signal due to the nuclear spin of $^{77}$Se was observed in the oxidized state, indicating that the electronic z-axis is perpendicular to the Ni-Se direction.

*Keywords:* [NiFe]hydrogenase; EPR; purification; selenium; selenocysteine.

The simplest biochemical redox reaction, namely the reversible, heterolytic cleavage of dihydrogen into two protons and two electrons is catalysed by enzymes called hydrogenases [1, 2]. These are found in many organisms from all three domains, bacteria, eukarya and archaea. Different classes are defined according to the metal content of the enzymes. One class comprises hydrogenases containing no metal other than iron. These enzymes are called Fe-only hydrogenases or [Fe]hydrogenases [1]. The second class consists of hydrogenases that contain nickel in addition to iron. Accordingly these enzymes were named [NiFe]hydrogenases. Most of the characterized hydrogenases belong to this group. The EPR spectra of nickel respond to redox changes [4–7] and this ion is part of the active site [8]. Some [NiFe]hydrogenases are known to possess selenium in the form of selenocysteine [9, 10]. These enzymes form a subclass of the [NiFe]hydrogenases. Such [NiFeSe]hydrogenases are found in *Desulfovibrio baculatus* [11], *Desulfovibrio salexigens* [12], *Methanococcus vannielii* [9], *Methanococcus voltae* [10] and, as predicted from the genome sequence, in *Methanococcus jannaschii* [13].

*M. voltae* is an anaerobic archaeon that gains energy by oxidation of hydrogen via hydrogenases and concomitant reduction of carbon dioxide to methane. Four gene clusters predicted to code for hydrogenases have been found in *M. voltae* [14]. The structure of the gene clusters indicate that two enzymes are able to reduce the electron acceptor F420, a deazaflavin, whereas no natural electron acceptor is known for the other two gene products. Accordingly the enzymes are called F420-reducing hydrogenases or F420-non-reducing hydrogenases. One hydrogenase of each type contains selenium in the form of selenocysteine [10, 15].

Depending on the redox potential, an unpaired electron, residing on the nickel ion, can be detected with EPR spectroscopy. [NiFe]hydrogenases have been purified aerobically. Such oxidized preparations are inactive but can be reactivated upon reduction. The oxidized preparations usually show two EPR signals in various ratios [5, 16–22]. One signal has $g$ values of 2.33, 2.16 and 2.01. Enzyme molecules with such a signal are readily activated upon exposure to hydrogen. Hence this state is termed 'ready' [17, 18]. We will refer to the active site in this state as $Ni_r$(III) (formerly called Ni-a or Ni-B). The other signal has $g$ values of 2.31, 2.23 and 2.02; molecules exhibiting this signal need a prolonged contact with hydrogen, often at elevated temperature, before activity is obtained. We therefore call the active site in this state $Ni_u$(III) (formerly called Ni-b or Ni-A).

Some hydrogenases have been purified anaerobically [10]. These preparations are active without further treatment. The anaerobically purified F420-non-reducing hydrogenase from *M. voltae* has been probed with EPR spectroscopy [23, 24]. This

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*Abbreviations.* I, nuclear spin; S, electron spin.

enzyme, as isolated under an atmosphere of 5% $H_2$ and 95% $N_2$, showed an EPR signal with $g$ values of 2.21, 2.15 and 2.01. This spectrum resembles a signal which was obtained upon reductive activation of oxidized hydrogenases from different origins [4, 17–19, 25, 26]. The species exhibiting this signal are light sensitive at low temperatures [23, 25]. Illumination with white light led to disappearance of the signal, and concomitantly a new signal with its lowest $g$ value at 2.05 was observed. It has been shown that the velocity of these light-sensitive changes was dependent on the bulk-hydrogen isotope provided by the solvent [25]. It was concluded by the authors that a photolytic cleavage of a nickel-hydrogen bond was responsible for the changes in the spectrum. All enzymes exhibiting these characteristics were catalytically active. Since in this state the active site is two reducing equivalents more reduced than the oxidized state we denote enzymes in this state $Ni_a(I)\times H_2$ prior to illumination and $Ni_a(I)[H_2]$ after illumination. In the literature these states are also called Ni-C and Ni-C* (or Ni-L), respectively.

Recent advances in the characterization of the active site by Mössbauer spectrocopy [27], X-ray diffraction [28] and Fourier-infrared spectroscopy [29–31] showed that the active site of [NiFe] hydrogenases consists of a bimetallic Ni-Fe site. The Fe ion is low spin and has an octahedral coordination with 5–6 ligands: Two cysteine thiols and an oxygen atom [28, 32], bridging between iron and nickel, plus two $CN^-$ and one CO molecules [31]. The nickel ion has five ligands in an octahedral coordination: three bridging ligands described above and two thiols [28, 32]. As the $\nu$(CN) and $\nu$(CO) stretch vibrations respond to redox and other changes in the active site [29], redox changes on the iron ion probably occur [32]. Hence the notations we use here are only valuable as a label to indicate the actual redox state of the active site as a whole, but not nescessarily that of the nickel ion, and is merely used for the sake of a clear discussion.

The $Ni_a(I)$ states of the selenium-containing F420-non-reducing hydrogenase from *M. voltae* have been extensively studied by means of EPR spectroscopy [23, 24]. In the $Ni_a(I)\times H_2$ state of $^{77}$Se-enriched enzyme an anisotropic hyperfine interaction of the nickel-based unpaired electron with the nuclear spin of the isotope was observed, showing that the selenium atom is a ligand of the nickel [23]. After illumination a hyperfine interaction was still observable but the splitting was nearly isotropic. Since the unpaired electron of the nickel ion in the 'dark' resided in an orbital with mainly $d_z2$ character and in an orbital with mainly $d_{x^2-y^2}$ character after illumination, and since the $^{77}$Se nucleus interacted in both states with this electron, it was assumed that the electronic z-axis could flip by 90° upon illumination.

In CO-treated enzyme the $g$ values of the signal differed from the $g$ values obtained from untreated enzyme [24]. As in hydrogenase from *Chromatium vinosum* [33] the nuclear spin of $^{13}$CO introduced an isotropic hyperfine splitting [24]. Although the EPR signal of the CO-treated species shows that the unpaired electron is in an orbital with mainly $d_z2$ character, both isotopes, $^{13}$CO and $^{77}$Se, introduced an hyperfine splitting. After illumination the interaction with the $^{13}$C nucleus was no longer observed but the splitting due to $^{77}$Se remained, although it was weaker [24]. From these results it was concluded that selenium and CO bind on opposite sides of the nickel and that the electronic z-axis flips by 90° upon photodissociation of the nickel-bound CO.

The F420-non-reducing hydrogenase from *M. voltae* has a peculiar feature. It contains an unusually small subunit of 25 amino acids [10]. The sequence of this subunit is similar to the highly conserved C-terminal sequence of the large subunit of other hydrogenases [14]. It contains selenium in the form of selenocysteine [10]. This selenium has been shown to be a ligand of the nickel in the active center [23]. The structure of a sulfur analogue of this subunit has been analysed and a nickel-binding capacity has been shown *in vitro* [34].

As the peculiar characteristics of the selenium splitting were observed only in the F420-non-reducing hydrogenase from *M. voltae* [23, 24] containing the unique small subunit, it could be argued that this particular enzyme might not be representative for other [NiFe]hydrogenases. To test whether the electronic structure around the nickel is similar in a selenium-containing hydrogenase where all cysteine-thiol ligands are present in one subunit, it was desirable to perform EPR measurements with the F420-reducing hydrogenase from *M. voltae*, which is predicted to harbour a selenocysteine at a conserved position [14]. To be able to prepare active enzyme in an amount suitable for spectroscopic investigations, an anaerobic purification procedure was established. We prepared samples with natural selenium and enriched in $^{77}$Se. No information was available on the possible interaction of the nickel-based unpaired electron and the selenium nucleus in oxidized enzyme. To address this question we have prepared oxidized samples of the selenium-containing F420-non-reducing and the selenium-containing F420-reducing hydrogenase from *M. voltae* with either natural selenium or $^{77}$Se.

## MATERIALS AND METHODS

**Growth of *M. voltae*.** *M. voltae* PS (DSM 1537) was grown at 37 °C in 10 l batch cultures with a $H_2/CO_2$ (80:20) gas phase. The growth medium described by Whitman et al. [35] was used with the following modifications. Cysteine and sodium sulfide were omitted. A small amount of $H_2S$ was added to the gas phase as a reductant and the sulfur source. 1 μM selenite, as the natural isotope mixture or enriched in $^{77}$Se (92.4 atom%; Promochem), was added. [$^{77}$Se]selenite was prepared as described earlier [23]. The selenium concentration in the medium without selenite addition was lower than 3 nM as determined by hydride atomic-absorption spectroscopy (Biodata). On this basis the $^{77}$Se enrichment was calculated to be 92%. Cells were harvested anaerobically during late exponential growth, frozen in liquid nitrogen, and stored at −80 °C.

**Isolation of the F420-reducing hydrogenase.** All purification steps were performed under exclusion of oxygen. Column chromatography and concentration of enzyme solutions were performed in an anaerobic glove box (Coy Laboratories) containing 5% $H_2$ and 95% $N_2$. Outside the glove box, solutions were stored in glass bottles sealed with rubber stoppers. Samples were added by means of gas tight syringes.

The hydrogen-uptake activity was determined in cuvettes sealed with rubber stoppers under a hydrogen atmosphere with benzyl viologen or F420 as electron acceptor. The reduction of the electron acceptor was monitored photometrically at 578 nm or 405 nm, respectively. The oxidation of 1 μmol $H_2$/minute is defined as 1 U.

**Crude lysate and centrifugation.** 10 g frozen cells were lysed in 30 ml buffer A (50 mM Tris/HCl, pH 7.5) containing 100 μg/ml DNAse by stirring at 18 °C for 30 min. Centrifugation for 30 min at 160000×$g$ yielded the S160 supernatant.

**Column chromatography.** The S160 fraction was applied to a DEAE-Sepharose fast-flow column (2.6 cm×16 cm; Pharmacia) and eluted with a step gradient of 0, 0.2, 0.25, 0.3, 0.35, 0.4 and 2 M NaCl in buffer A. The fractions exhibiting F420-reducing activity were pooled and 4 M ammonium sulfate was added to 0.7 M. This solution was applied to a phenyl-Superose HR 10/10 column (Pharmacia). After a wash with 0.7 M ammonium sulfate the column was developed with 0.5, 0.25 and 0 M ammonium sulfate in buffer A. The F420-reducing activity

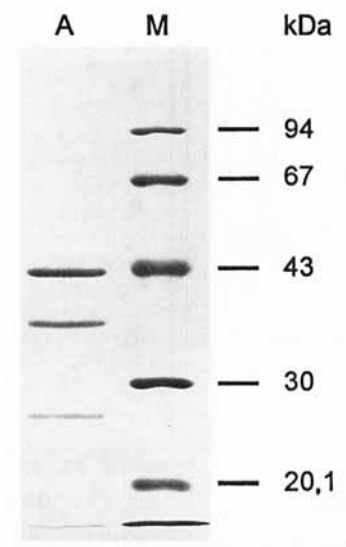


**Fig. 1. Gel electrophoretic analysis of the purified F420-reducing hydrogenase.** The subunits of the purified enzyme were separated on a 12% SDS gel according to Laemmli [44]. Lane A, purified enzyme. Three bands with apparent molecular masses of 45, 37, and 27 kDa can be seen. M, size marker.

eluted with 0.25 M ammonium sulfate. The volume of this fraction was reduced by ultrafiltration through an Omega membrane (Filtron) and the sample was diluted fivefold with buffer A to reduce the salt concentration. This solution was loaded on a Mono Q HR 10/10 column (Pharmacia). After washing with buffer A alone and with 0.24 M NaCl in buffer A, the protein was eluted with a 70-ml linear gradient from 0.24 M to 0.44 M NaCl in buffer A. The active fraction was concentrated by ultrafiltration by means of Centricon 30 (Amicon) microconcentrators.

**Isolation of the F420-non-reducing hydrogenase.** The F420-non-reducing hydrogenase was purified as reported previously [10]. Enzyme solutions were concentrated by ultrafiltration through Centricon 30 microconcentrators (Amicon).

**Preparation of samples for EPR spectroscopy.** The concentrated hydrogenase solutions were anaerobically transferred into EPR tubes and quickly frozen. Oxidized samples were generated by opening the EPR tubes at room temperature, whereafter equilibration with air was established by flicking the tube. After an incubation of 2 min the tubes were frozen.

**EPR spectroscopy.** EPR spectra at X band (9 GHz) were obtained with a Bruker ECS 106 EPR spectrometer at a field-modulation frequency of 100 kHz. Cooling of the sample was performed with an Oxford Instruments ESR 900 cryostat with an ITC4 temperature controller. The magnetic field was calibrated with an AEG Magnetic Field Meter. The X-band frequency was measured with a HP 5350B Microwave Frequency Counter. Illumination of the samples was carried out in the helium cryostat by shining white light (Osram Halogen Bellaphot, 150 W) via a light guide through the irradiation grid of the Bruker ER 4102 ST cavity. Dark adaption of illuminated samples was carried out by placing the samples for 10 min in cold (200 K) ethanol in the dark. For computer simulations the program EPR [Neese, F. (1995) Quantum chemistry program exchange, Indiana, USA, program no. QCMP136] was used. Normalized double-integral values (i.e. the spin concentrations) of the simulated spectra were calculated and used to determine the weights neccessary for the simulation of the various spectra.

## RESULTS

**Purification of F420-reducing hydrogenase from *M. voltae*.** To obtain highly active protein in quantities, which allow subsequent spectroscopic investigations of the enzyme, a purification procedure under anoxic conditions was used. The enzyme was purified to apparent homogeneity as determined via SDS/PAGE (Fig. 1). The specific hydrogen-uptake activity of the purified enzyme was 350 U/mg protein (Table 1). This value is higher than the value reported for the aerobically purified enzyme, which was 10 U/mg protein [36]. The yield was calculated to be 7.4%. The subunits, as separated on a denaturing gel had apparent molecular masses of 45, 37 and 27 kDa, in reasonable accordance with masses derived from the gene sequences. Bands of the same apparent molecular mass have been reported for the aerobically purified enzyme [36]. However, a band of an apparent molecular mass of 55 kDa, as previously reported, was not observed with the present preparations. It has been argued that this band might have been due to partially denatured protein [36]. No partially denatured protein was observed with our preparations after 5-min boiling with SDS and dithiothreitol.

**EPR spectra of the active site in F420-reducing hydrogenase as isolated.** Since the anaerobically performed purification yielded large amounts of highly active enzyme, it was possible to record EPR spectra of this hydrogenase. In addition to signals of Fe-S clusters observed at low temperatures (data not shown), the enzyme under 5% of hydrogen showed a rhombic EPR spectrum at 70 K with $g_{xyz}$ = 2.21, 2.15 and 2.01. The latter line was obscured by radical species in the sample (Fig. 2IA) and could only be detected in difference spectra. The $g$ values are typical for selenium-containing [NiFe]hydrogenases in the $Ni_a(I)\times H_2$ state [23, 26]. As in other hydrogenases, the species responsible for this signal was light sensitive at low temperatures. Illumination below 77 K led to disappearance of this signal and the appearence of another signal with $g_{xyz}$ values of 2.05, 2.11, and 2.29 (Fig. 2IB). This signal resembles the light-induced signal of the F420-non-reducing hydrogenase from *M.*

**Table 1. Purification of the F420-reducing hydrogenase from *M. voltae*.** The electron acceptors used for the activity determination are given. Specific activities were calculated for the reduction of F420. BV, benzyl viologen.

| Fraction | Activity | | Protein | Yield | Purification factor | Specific activity |
|---|---|---|---|---|---|---|
| | BV | F420 | | | | |
| | U | | mg | % | -fold | U/mg |
| S160 | 592350 | 29580 | 1550 | 100 | 1 | 19 |
| DEAE-Sepharose | 70220 | 4660 | 66 | 15.8 | 3.7 | 71 |
| Phenyl-Superose | 6610 | 2180 | 13 | 7.4 | 8.8 | 168 |
| Mono Q | 1620 | 2200 | 6.3 | 7.4 | 18.4 | 349 |

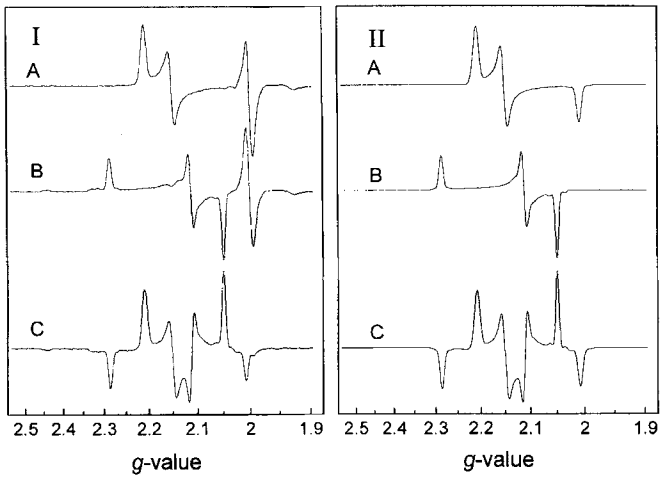


**Fig. 2. EPR spectra of F420-reducing hydrogenase.** (I) Experimental spectra. The purified protein was frozen in the dark without further treatment. (A) Spectrum of the sample prior to illumination. (B) Spectrum of the same sample after 5 min illumination with white light at 70 K. (C) Difference spectrum of the spectra shown in (A) and (B). EPR conditions: microwave frequency, 9420.4 MHz; microwave power incident to the cavity, 2.6 mW; modulation amplitude, 1.27 mT; temperature, 70 K. (II) Simulations of the spectra shown in (I). (A) Simulation of the $Ni_a(I)\times H_2$ signal of the F420-reducing hydrogenase. (B) Simulation of the $Ni_a(I)[H_2]$ signal. (C) Difference of the simulations shown in (A) and (B). Parameters used: microwave frequency, 9420.4 MHz. (A) Summation of two spectra with (a) $g_{xyz}$ = 2.207, 2.149, 2.0075; $w_{xyz}$ = 0.972, 0.919, 0.848 mT; weight, 0.9242 [$Ni_a(I)\times H_2$ without $^{77}Se$] and (2) $g_{xyz}$ = 2.207, 2.149, 2.0075; $w_{xyz}$ = 0.972, 0.919 with a hyperfine interaction, $I$ = 1/2, $A_{xyz}$ = 0.97, 1.79, 5.39 mT; weight, 0.0758 [$Ni_a(I)\times H_2$ with 100% $^{77}Se$]. (B) Summation of two spectra with (1) $g_{xyz}$ = 2.0478, 2.1099, 2.285; $w_{xyz}$ = 0.583, 0.707, 0.730 mT; weight, 0.9242 {$Ni_a(I)[H_2]$ without $^{77}Se$} and (2) $g_{xyz}$ = 2.0478, 2.1099, 2.285; $w_{xyz}$ = 0.583, 0.707, 0.730 mT; with a hyperfine interaction, $I$ = 1/2; $A_{xyz}$ = 4.36, 4.79, 4.01 mT; weight, 0.0758 {$Ni_a(I)[H_2]$ with 100% $^{77}Se$}.

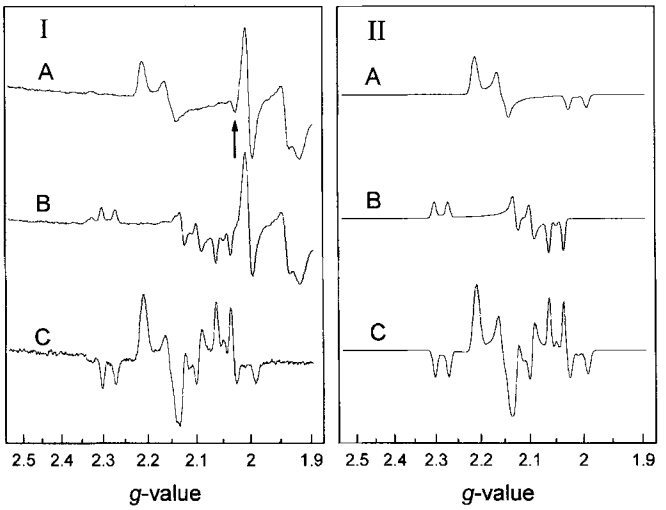


**Fig. 3. EPR spectra of $^{77}Se$-enriched F420-reducing hydrogenase as isolated.** (I) Experimental spectra: The purified protein was frozen in the dark without further treatment. (A) Spectrum of the sample prior to illumination. The arrow points to a trough that is due to the split $g_z$ line of the $Ni_a(I)\times H_2$ signal. (B) Spectrum of the same sample after 5 min illumination with white light at 70 K. (C) Difference of the spectra shown in (A) and (B). EPR conditions: microwave frequency, 9416.7 MHz; microwave power incident to the cavity, 2.6 mW; modulation amplitude, 1.27 mT; temperature, 70 K. (II) Simulations of the spectra shown in (I) (A) Simulation of the $Ni_a(I)\times H_2$ signal of the $^{77}Se$-enriched F420-reducing hydrogenase. (B) Simulation of the $Ni_a(I)[H_2]$ signal. (C) Difference of the simulations shown in (A) and (B). Parameters as in Fig 2II but for the microwave frequency (9416.7 MHz) and weights. The weights were as follows. For (A), $Ni_a(I)\times H_2$ without $^{77}Se$ =0.08, $Ni_a(I)\times H_2$ with 100% $^{77}Se$ = 0.92. For (B), $Ni_a(I)[H_2]$ without $^{77}Se$ = 0.08, $Ni_a(I)[H_2]$ with 100% $^{77}Se$ = 0.92.

*voltae* [23] and is similar to the signal of the $Ni_a(I)[H_2]$ species of other hydrogenases [25, 37]. To resolve the high-field line of the 'dark' spectrum, a difference spectrum of the spectra (Fig. 2IA, B) was calculated (Fig. 2IC). Here the $g_z$ line of the 'dark' spectrum can be seen at $g$ = 2.01. All spectra shown in Fig. 2I could be simulated as $S$ = 1/2 spectra and are shown in Fig. 2II. The natural abundance of $^{77}Se$ (7.58%) was taken into account. Thus each simulated spectrum shown in Fig. 2II consists of two simulations, namely, one assuming no $^{77}Se$ and one assuming 100% $^{77}Se$. The hyperfine tensors neccessary to calculate the latter simulations were determined from the spectra of the $^{77}Se$-enriched sample.

**EPR spectra of $^{77}Se$-enriched F420-reducing hydrogenase as isolated.** As outlined above, the F420-reducing hydrogenase was predicted to contain selenium in the form of selenocysteine. Due to the conserved position of this selenocysteine it is highly likely that the selenium is a ligand to the nickel, as has been shown for other selenium-containing hydrogenases [23, 27]. This question was addressed by preparing enzyme enriched in $^{77}Se$. The spectrum of such a preparation (prepared under 5% hydrogen) is shown in Fig. 3IA. Comparison with Fig. 2IA shows that the $g_x$ and $g_y$ lines at $g$ = 2.21 and 2.15 are broadened. This is ascribed to the hyperfine interaction of the unpaired electron of the nickel with the nuclear spin of the $^{77}Se$. The high-field line is overlapping with a radical signal as in the preparation containing natural selenium. However, careful inspection of this region shows a trough (Fig. 3IA) that is absent in the sample with natural selenium. This indicates a large splitting of the $g_z$ line due to $^{77}Se$. After illumination of the sample, the spectrum in Fig. 3IB was observed. All the lines of the 'light' spectrum are split twofold due to the interaction with the nuclear spin of the $^{77}Se$. Fig. 3IC shows the difference spectrum of the 'dark' and 'light' spectrum of the $^{77}Se$-enriched sample. From this spectrum it can be confirmed that the trough in the 'dark' spectrum (Fig. 3IA) is part of the split $g_z$ line of this signal. Thus, the splitting introduced by $^{77}Se$ is highly anisotropic in the 'dark' signal. In contrast it is nearly isotropic after illumination.

The spectra shown in Fig. 3I could be simulated as $S$ = 1/2 spectra using the same parameters as in Fig. 2II but with different weights for the individual spectra. The simulations are shown in Fig. 3II. Comparison with the experimental spectra shows a good fit. The hyperfine tensors determined by the simulation confirm the notion that the splitting is highly anisotropic in the 'dark' spectrum ($A_{xyz}$ = 0.97, 1.79 and 5.39 mT) and nearly isotropic after illumination of the sample ($A_{xyz}$ = 4.36, 4.79 and 4.01 mT).

**EPR spectra of oxidized F420-reducing hydrogenase.** Purified F420-reducing hydrogenase was oxidized by exposing the sample to air. Such a sample showed an EPR spectrum as in Fig. 4IA. The low-field lines of the rhombic spectrum can be detected at $g$ = 2.41 and 2.17. The high-field line, which is expected at $g$ = 2.01, was obscured due to overlap with a radical signal. In view of the $g$ values, the signal is attributed to the 'ready' species [$Ni_r$(III)] although the $g_x$ value is considerably higher than in other [NiFe]hydrogenases. To investigate the influence of $^{77}Se$ on this spectrum, samples enriched in $^{77}Se$ were prepared. The EPR spectrum of such a sample is shown in Fig. 4IB. Comparison of the line shape of these two spectra

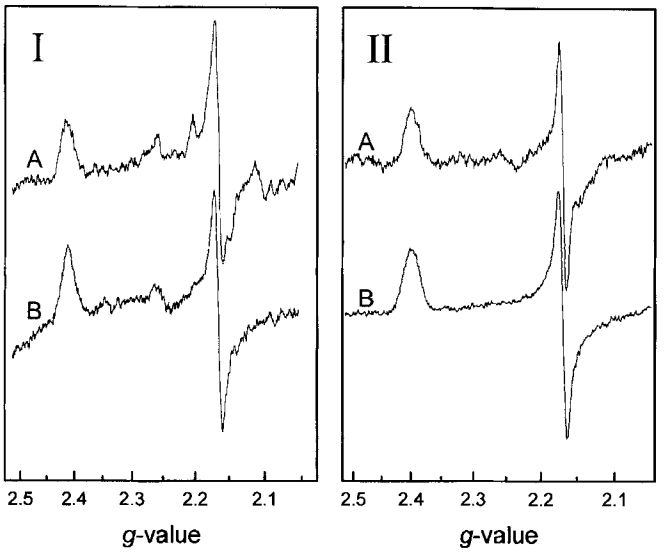


**Fig. 4. EPR spectra of oxidized hydrogenase containing natural selenium and enriched in $^{77}$Se.** The purified enzymes were oxidized by exposure to air. EPR conditions: microwave power incident to the cavity, 2.6 mW; modulation amplitude, 1.27 mT; temperature, 12.5 K. (I) Spectra of F420-reducing hydrogenase. (A) Spectrum of a sample containing natural selenium. (B) Spectrum of a sample enriched in $^{77}$Se. Microwave frequency, 9415 MHz. (II) Spectra of F420-non-reducing hydrogenase. (A) Spectrum of a sample containing natural selenium. (B) Spectrum of a sample enriched in $^{77}$Se. Microwave frequency, 9416.3 MHz (A), 9415.1 MHz (B).

shows no apparent effect of $^{77}$Se. This means that the hyperfine interaction of the nickel-based unpaired electron with the $^{77}$Se nucleus must be considerably smaller than the line width.

**EPR spectra of oxidized F420-reducing hydrogenase.** Oxidized F420-non-reducing hydrogenase was prepared by treatment with air. The spectrum of such a sample is displayed in Fig. 1 IIA. This spectrum with its low-field lines at $g = 2.397$ and 2.171 can be attributed to the $Ni_r$(III) species, similarly to the spectrum obtained with oxidized F420-reducing hydrogenase. The $g_z$ line expected at $g = 2.01$ is obscured by overlapping signals. To test wether the influence of $^{77}$Se on this spectrum is comparable to the influence observed for the F420-reducing enzyme, the F420-non-reducing hydrogenase was prepared from cells grown in $^{77}$Se-enriched medium. After oxidation, such samples gave spectra as displayed in Fig. 4 IIB. With these concentrated samples only minor effects of $^{77}$Se, the largest being an apparent 0.63-mT broadening of the $g = 2.17$ line, were observed. The $g = 2$ region was studied thoroughly. No signs of a possible splitting of the $g_z$ line was observed (data not shown).

## DISCUSSION

**Properties of the F420-reducing hydrogenase.** The F420-reducing hydrogenase from *M. voltae* has been anaerobically purified to a specific hydrogen-uptake activity of 350 U/mg with F420 as electron acceptor. This value is 35-fold higher than the value obtained for aerobically purified enzyme [36]. The specific activity of hydrogenases was about 80-fold higher in extracts of cells where contact with oxygen was minimized, compared with cells harvested and disrupted in air. This indicates that oxygen affects the activity irreversibly. In addition, a polypeptide of a molecular mass of 55 kDa observed in previous preparations was probably a contamination since it has not been found in the highly active preparations shown in this article.

**EPR characteristics of the enzyme.** The enzyme as isolated under 5% hydrogen was EPR active and showed spectra characteristic for enzymes in the $Ni_a$(I) state. The $g$ values were almost identical to the $g$ values reported for the F420-non-reducing hydrogenase from the same organism [23] and similar to the $g$ values reported for other [NiFe]hydrogenases [25, 26, 37]. Light-sensitive changes typical for [NiFe]hydrogenases in this state were observed.

Enrichment in $^{77}$Se resulted in an anisotropic splitting of the $Ni_a$(I)×$H_2$ signal by the nuclear spin ($I = 1/2$) of the $^{77}$Se nucleus. The hyperfine tensors ($A_{xyz} = 0.97$, 1.79 and 5.39 mT) are in the same range as the previously reported values from the F420-non-reducing enzyme ($A_{xyz} = 0.96$, 1.548 and 5.32 mT) [23] and the estimated values of $A_x$ and $A_y$ of the [NiFeSe]hydrogenase from *D. baculatus* (1.0 mT and 1.8 mT, respectively) [26]. The strong splitting of the $g_z$ line ($A_z = 5.39$ mT) and the much smaller splitting in the $g_x$ and $g_y$ lines ($A_{xy} = 0.97$ mT and 1.79 mT) indicate that mainly a dipole-dipole interaction contributes to the overall splitting. Since the $g$ values ($g_z$ close to $g_e$, and $g_{xy} > g_z$) are characteristic for an unpaired $d$ electron in an orbital with a large $d_z2$ contribution [38], it is concluded that the selenium atom is a ligand to nickel and that the electronic z-axis is along the Ni-Se bond.

After illumination, the signal changed considerably but the $^{77}$Se nucleus still interacted with the unpaired electron. As observed for the F420-non-reducing hydrogenase [23], the splitting was nearly isotropic ($A_{xyz} = 4.36$, 4.79 and 4.01 mT) indicative for spin density on the selenium nucleus, presumably due to $s$ orbital contribution. Since in the $Ni_a$(I)[$H_2$] species no $g$ value is close to $g_e$, it is assumed that the unpaired electron is no longer in an orbital with mainly $d_z2$ character but rather in an orbital with most likely $d_{x^2-y^2}$ character. In contrast to the $d_z2$ orbital, this orbital has a planar symmetry. Thus the interaction of the unpaired electron with the selenium nucleus can be explained by assuming a 90° flip of the electronic z-axis, as proposed earlier [23]. A change of the geometric position of the selenium atom is unlikely since the light-sensitive changes can be observed at temperatures down to 4.2 K and the selenium is part of a selenocysteine in the polypeptide chain and therefore fixed in the rigid spatial structure of the protein.

These results compare very well to previously performed experiments with the F420-non-reducing hydrogenase from *M. voltae* [23], suggesting that the observed characteristics are common for [NiFe]hydrogenases.

**EPR characteristics of the oxidized enzyme.** Up to now no data on selenium-containing hydrogenases were available in one of the oxidized states. The F420-reducing hydrogenase when oxidized under air showed a spectrum (Fig. 4 IA) which can be attributed to a state of the enzyme in which it can readily be activated [$Ni_r$(III)]. Such a spectrum was also observed when the enzyme was oxidized with dichloroindophenol (Pfeiffer, M., unpublished results).

The $g$ value of the low-field line ($g_x = 2.41$) is higher than the value reported for most other hydrogenases, which is about 2.33 [33, 39]. The $g_y$ line at $g = 2.17$ is at a position where it is found in most other enzymes.

The F420-non-reducing hydrogenase showed a similar spectrum in its oxidized state (Fig. 4 IIA). This shows that the structure of these two enzymes around the nickel is similar despite that the F420-non-reducing hydrogenase contains part of the nickel-binding site on a seperate subunit [10, 23, 34].

The spectra of the $^{77}$Se-enriched enzymes (Fig. 4IB, IIB) showed no detectable splitting due to $^{77}$Se. Inspection of the $g_z$ region gave no indication for a possible splitting due to $^{77}$Se.

In hydrogenase from *Wolinella succinogenes* enriched in $^{33}$S, an influence of the isotope was detectable in the oxidized [$Ni_r$(III)] and in the reduced [$Ni_a$(I)] states [39]. The observed effect was explained by an interaction of the unpaired electron of the nickel with one sulfur atom, but not necessarily the same one, in both oxidation states. The effective hyperfine interaction of $^{77}$Se is usually larger than that of $^{33}$S. Although the magnetic dipole moments of both nuclei are comparable [38], the energies of the 4*s* and 4*p* orbitals of selenium are considerably higher than those of the 3*s* and 3*p* orbitals of sulfur. This favours orbital overlap with the metal 3*d* orbitals [40]. Therefore the splitting due to $^{77}$Se is expected to be larger than the splitting caused by $^{33}$S if the structure of the active site is comparable. The observed effects for the $Ni_a(I)\times H_2$ and $Ni_a(I)[H_2]$ states are consistent with this assumption. Therefore, we conclude that the sulfur atom that caused the hyperfine effect in the $Ni_a(I)\times H_2$ state of $^{33}$S-enriched enzyme from *W. succinogenes* has the same geometrical position as the selenium atom in selenium-containing hydrogenases. In accordance with this, the largest hyperfine effect was observed at $g_z$ in both cases. The quality of the spectra of the $Ni_a(I)[H_2]$ state of the $^{33}$S preparation from *W. succinogenes* [39] does not permit a proper analysis on this point.

The $Ni_r$(III) signal has one of its $g$ values close to $g_e$, suggesting that the unpaired electron is in an orbital with predominantly $d_z2$ character, similar to that in the $Ni_a(I)\times H_2$ state. In $^{77}$Se-enriched enzyme the unpaired electron in the $Ni_a(I)\times H_2$ state experienced a hyperfine interaction of 5.39 mT in the z-direction, which was the Ni-Se axis. In contrast, no apparent effect was detected in the $Ni_r$(III) state. Therefore we conclude that the electronic z-axis is perpendicular to the Ni-Se direction. This means that the electronic z-directions in oxidized and reduced enzymes are perpendicular to each other. This in turn might reflect a considerable difference in coordination around nickel.

Taking these results into account it follows that the sulfur atom that caused the hyperfine interaction in oxidized hydrogenase from *W. succinogenes* [39] is not the one at the position of the selenium atom in selenium-containing hydrogenases, but must be the one with a Ni-S bond perpendicular to the Ni-Se direction. In the nomenclature of the *Desulfovibrio gigas* enzyme [28], this means that it must be the thiol of Cys65 or Cys533. Van der Zwaan et al. [33] have shown that reoxidation of reduced hydrogenase from *C. vinosum* with $^{17}$O-enriched $O_2$ led to a slight broadening of EPR signals of $Ni_r$(III) and $Ni_a$(III) by $^{17}$O ($I$ = 5/2). Volbeda et al. [32] found evidence for a non-protein bridging ligand between Ni and Fe in the oxidized *D. gigas* enzyme and consider it reasonable to assume that this may be an oxygen atom. If the $d_z2$ orbital of the nickel, holding the unpaired spin, would point to the S atom of Cys65, it would also point to the putative O atom and a larger hyperfine effect would have been expected. We therefore favour the Ni-S(Cys533) direction as the z-direction in the oxidized enzyme. Direct measurement of the $g$ tensor in crystals of oxidized [NiFe] hydrogenase from *Desulfovibrio vulgaris*, Miyazaki F [41] resulted in the Ni-S(Cys533) direction as the z-axis.

X-ray−absoption-spectroscopy studies on different hydrogenases in various oxidation states indicated that the charge density on the nickel does not change significantly [42, 43]. Two explanations were given for this observation. One possibility would be that the redox chemistry takes place at another redox component. In view of the knowledge that the active site is composed of a dinuclear cluster of iron and nickel [27, 28, 31, 32] it is conceivable that the iron undergoes most of the redox changes, as is suggested by the frequency shift of the $\nu$(CN) and $\nu$(CO) stretch vibrations [31]. Via spin-spin coupling this could make the unpaired spin of the nickel either EPR detectable if the iron ion is diamagnetic, or undetectable if the iron ion is paramagnetic (in the $S$ = 1/2 state). The unpaired spin of the nickel does not interact noticeably with the $^{57}$Fe nucleus in the $^{57}$Fe-enriched enzyme from *C. vinosum* as monitored with S-band EPR or X-band electron nuclear double-resonance spectroscopy (Gessner, C., Roseboom, W., Albracht, S. P. J. and Lubitz, W., unpublished observations). Another explanation was the assumption that the redox chemistry could take place on the ligands of the nickel rather than on the nickel itself. The unpaired spin observed with EPR spectroscopy experiences a hyperfine interaction caused by $^{77}$Se, ranging from 12.1 mT in CO-treated enzyme [24] to a nearly undetectable effect in oxidized enzyme. This indicates that the spin density on the $^{77}$Se nucleus differs between these states. This might be in line with the assumption that the selenium is involved in redox chemistry in that it buffers charges by delocalization. This might be tested by X-ray−absorption-spectroscopy studies focussing on the absorption edge of the selenium. Such experiments are under way.

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