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Influence of illumination on the electronic interaction between ^{77}Se and nickel in active F_{420}-non-reducing hydrogenase from *Methanococcus voltae*

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The selenium-containing F_{420}-non-reducing hydrogenase from *Methanococcus voltae* was anaerobically purified. The enzyme as isolated showed an EPR spectrum with $g_{x,y,z}$ = 2.21, 2.15 and 2.01. Upon illumination this spectrum disappeared and a new signal with the lowest g value at 2.05 arose. EPR studies were carried out either with the enzyme containing natural selenium or enriched in the nuclear isotope ^{77}Se. The hyperfine splitting caused by ^{77}Se in the 'dark' signal is shown to be highly anisotropic. In contrast the splitting is nearly isotropic after illumination. A new model for the nickel site is proposed to explain these observations.

EPR; ^{77}Se; Nickel; Hydrogenase; *Methanococcus voltae*

1. INTRODUCTION

Hydrogenases catalyse the reversible oxidation of dihydrogen to two protons and two electrons. Hydrogenases are divided into different classes according to their metal content. One group consists of the enzymes possessing only iron. These are called Fe-only hydrogenases or [Fe] hydrogenases [1]. Hydrogenase I from *Clostridium pasteurianum* [2] and the periplasmic hydrogenase from *Desulfovibrio vulgaris* strain Hildenborough [3,4] belong to this group. Other hydrogenases contain nickel in addition to iron. These enzymes are called [NiFe] hydrogenases. Enzymes of this type are found in many prokaryotic organisms [5–7]. Some hydrogenases are known to possess selenium in the form of selenocysteine. This was first deduced from the detection of radioactive derivatives of alkylated selenocysteine obtained from hydrolysed, ^{75}Se-labeled hydrogenase from *Methanococcus vannielii* [8]. It was recently conclusively demonstrated via laser desorption mass spectrometry with the 3 kDa subunit of the F_{420}-non-reducing hydrogenase from *Methanococcus voltae* [9]. The [NiFeSe] hydrogenases form a subclass of the [NiFe] hydrogenases. Enzymes of this type are found in *M. vannielii* [8], *Desulfovibrio baculatus* [10–12] and *M. voltae* [9, 13]. EPR spectra on ^{77}Se-enriched (I = 1/2) hydrogenase from *D. baculatus* [11] and also EXAFS measurements [14] showed that selenium is a direct ligand to nickel. Recently a hydrogenase was discovered in *Methanobacterium thermoautotrophicum* [15] which appeared to lack any metal.

M. voltae is an anaerobic archaeon, which is able to gain energy by oxidation of hydrogen and concomitant reduction of carbon dioxide to methane. *M. voltae* possesses four gene clusters encoding two [NiFe] and two [NiFeSe] hydrogenases [16]. The structures of the respective gene clusters indicate that one hydrogenase of each type is able to reduce the electron acceptor F_{420} whereas the other is not. Accordingly the enzymes are termed F_{420}-reducing or F_{420}-non-reducing hydrogenase.

It is generally believed that the nickel ion is the hydrogen activating site in [NiFe] hydrogenases [5,6,17]. Hydrogenases are often oxygen-sensitive, but many [NiFe] hydrogenases have been purified under aerobic conditions. These hydrogenases exhibited two different EPR spectra in variable amounts [18–25]. One signal defined by g values of 2.31, 2.23 and 2.02 was termed Ni-A by some investigators and Ni-b by others [23]. Hydrogenases showing this signal are inactive and need prolonged reductive activation [19,20]. Therefore hydrogenases in this state are also denoted as 'unready' and we call the corresponding nickel here Ni_u(III) to avoid confusion. The second EPR signal, which is observable in aerobically purified hydrogenases, has g values of 2.33, 2.16 and 2.01. This signal, termed Ni-B by some workers and Ni-a by others, represents a form of the enzyme, that is readily activated upon exposure to hydrogen. Therefore hydrogenases in this state are called 'ready'

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Abbreviation: Coenzyme F_{420}, 7,8-didemethyl-8-hydroxy-5'-deazariboflavin

[19,20] and we call the corresponding nickel here Ni_r(III). During redox cycling a third species could be observed. This species, as first described by Orme-Johnson and coworkers [26], exhibits g values of 2.19, 2.14 and 2.02 [19,20,22,27] in [NiFe] hydrogenases and 2.23, 2.17 and 2.01 in a [NiFeSe] hydrogenase [11]. As this was the third form of nickel in hydrogenases to be detected in EPR, it was originally termed Ni-C. It represents an active form of the enzyme. As discovered by van der Zwaan et al. [27], the species responsible for the signal is light-sensitive. Upon illumination with white light at temperatures below 77 K, the Ni-C signal disappeared and a new signal with its lowest g value at 2.05 was observed [27]. As the nickel site in this state is two-reducing equivalents more reduced than Ni(III), we refer to the corresponding nickel as Ni_a(I). Van der Zwaan et al. [27–29] provided strong evidence that the Ni_a(I) can bind either hydrogen or CO at the same site.

According to van der Zwaan et al. [29] the unpaired spin in the 'dark' (unperturbed) $Ni_a(I)\cdot H_2$ state (Ni-C) resides in an orbital mainly with d_z2 character. This allows the unpaired spin to detect magnetic nuclei in the z direction in two ways: (i) via dipole–dipole interaction nearly exclusively resulting in a hyperfine splitting in the g_z (anisotropic splitting); (ii) via orbital overlap with ligand orbitals with an s-character, resulting in splittings in all directions (isotropic splitting). After photodissociation of the bound hydrogen, however, the g values report that the unpaired spin is no longer in a d_z2 orbital, but rather in a $d_{x^2-y^2}$ orbital (theoretically an unpaired spin in a d_{xy}, d_{yz} or d_{xz} orbital would also give rise to similar g values). In this case the unpaired spin might probe four ligand positions for possible magnetic nuclei. It is therefore regrettable that He and coworkers [11] did not provide information on the possible splitting in the z direction of the Ni-C signal. Also a most interesting experiment, namely detection and inspection of the signal expected to appear after illumination, was not reported.

In this paper we report experiments carried out with the F_{420}-non-reducing hydrogenase from *M. voltae* 92% enriched in ^{77}Se. The results considerably changed our view on the possible electronic structure of the nickel site and the coordination with hydrogen and CO.

2. MATERIALS AND METHODS

2.1. *Growth medium*

Medium according to Whitman et al. [30] was used with the following modifications. Cysteine, sodium selenite and sodium sulfide were omitted. H_2S was used as a reductant and the sulfur source. ^{77}Se (92.4 atom% ^{77}Se) was obtained from Promochem (Wesel, Germany). For oxidation to selenite elementary selenium, either the ^{77}Se-enriched isotope or the natural isotope mixture, was dissolved in concentrated nitric acid to give a 1 M solution. This solution was used to obtain 10 μM final concentration of selenite in the medium.

The selenium concentration in the medium without selenite addition was lower than 3 nM as determined by hydride atomic absorption spectroscopy. On this basis the ^{77}Se enrichment was calculated to be 92%.

2.2. *Growth of* M. voltae

M. voltae PS (DSM 1537) was grown at 37°C in 10 liter batch cultures. Cells were harvested anaerobically during exponential growth and stored at −80°C.

2.3. *Sample preparation*

Hydrogenase was purified as previously reported [9]. The purified hydrogenase was concentrated by ultrafiltration through centricon 30 microconcentrators (Amicon, Witten-Herdecke) and stored in liquid nitrogen.

2.4. *EPR spectroscopy*

EPR measurements 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 a 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. Simulation was carried out as has been described elsewhere [31]. 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 adaptation of the samples was obtained by placing them for 10 min in cold (200 K) ethanol in the dark prior to the measurement.

3. RESULTS

The active hydrogenase, as isolated in an anaerobic glove box containing 5% H_2, showed an EPR spectrum displayed in Fig. 1, trace A. The lines with the largest amplitudes are due to reduced Fe-S clusters (lines around $g = 2.05$ and $g = 1.95$) and radical species (line around $g = 2$) in the sample. At lower field, weak lines around $g = 2.21$ and 2.15 can be seen that appear to be due to the $g_{x,y}$ lines of the 'dark' form of $Ni_a(I)\cdot H_2$, i.e. the Ni-C signal. The g_z line is completely obscured by the strong overlapping lines due to the other paramagnets in the sample. As earlier demonstrated [32], the light sensitivity of the Ni-C signal makes it possible to obtain rather clear-cut EPR spectra of both the 'dark' and the 'light' (light-induced) version of the Ni_a(I) state, eliminating all background signals in the 'dark' minus 'light' difference spectrum. Upon illumination at 35 K the spectrum in Fig. 1, trace A rapidly changed (half life = 20 s; data not shown) and the spectrum in trace B was obtained. Fig. 1, trace C shows the difference spectrum 'dark' minus 'light'. In this spectrum the high-field lines of both the 'dark' $Ni_a(I)\cdot H_2$ species (Ni-C) and the 'light' Ni_a(I) species can be clearly detected at $g = 2.01$ and 2.05, respectively.

The resulting difference spectrum was verified by simulation. For this simulation the 7.58% natural abundance of ^{77}Se was taken into account. Thus, four basic simulations were required to obtain the final spectrum, namely two assuming no ^{77}Se interaction ('dark' and 'light' signals), and the two assuming 100% ^{77}Se interaction ('dark' and 'light' signals), whereby the superhyperfine-splitting values were obtained from the experi-

mental spectra from a ^{77}Se-enriched preparation (see below). The parameters are given in the legend to Fig. 1. The simulation (Fig. 1, trace D) is identical, within error, to the experimental difference spectrum, demonstrating that both spectra are due to simple $S = 1/2$ species.

Fig. 2. shows the spectra of the ^{77}Se-enriched hydrogenase. The high field lines of the 'dark' and the 'light' $Ni_a(I)$ signals were again undetectable due to overlap from signals of Fe-S clusters and a radical (traces A and B). They could be clearly seen, however, in a 'dark' minus 'light' difference spectrum (trace C). The enrichment in ^{77}Se resulted in a considerable broadening of the $g_{x,y}$ lines of the 'dark' $Ni_a(I)\cdot H_2$ signal, whereas the g_z line showed a clear two-fold splitting. Best-fitting simulations were obtained by invoking $I = 1/2$ superhyperfine splittings of 0.96, 1.548 and 5.32 mT in the x, y and z direction, respectively. The light-induced $Ni_a(I)$ species showed an EPR signal with resolved superhyperfine splittings of 4.327, 4.665 and 3.81 mT in the x, y and z direction, respectively.

4. DISCUSSION

The anaerobically purified F_{420}-non-reducing hydrogenase from *M. voltae* clearly shows EPR signals of the 'dark' $Ni_a(I)\cdot H_2$ species (Ni-C state) characteristic for all nickel hydrogenase inspected so far [6,27,29]. This corresponds to the observations that neither reductive activation of the enzyme was necessary prior to activity measurement, nor was a lag phase in activity observable. As in the *D. baculatus* enzyme [11] the EPR spectra shown here prove without any doubt that selenium is a ligand to the redox-active nickel.

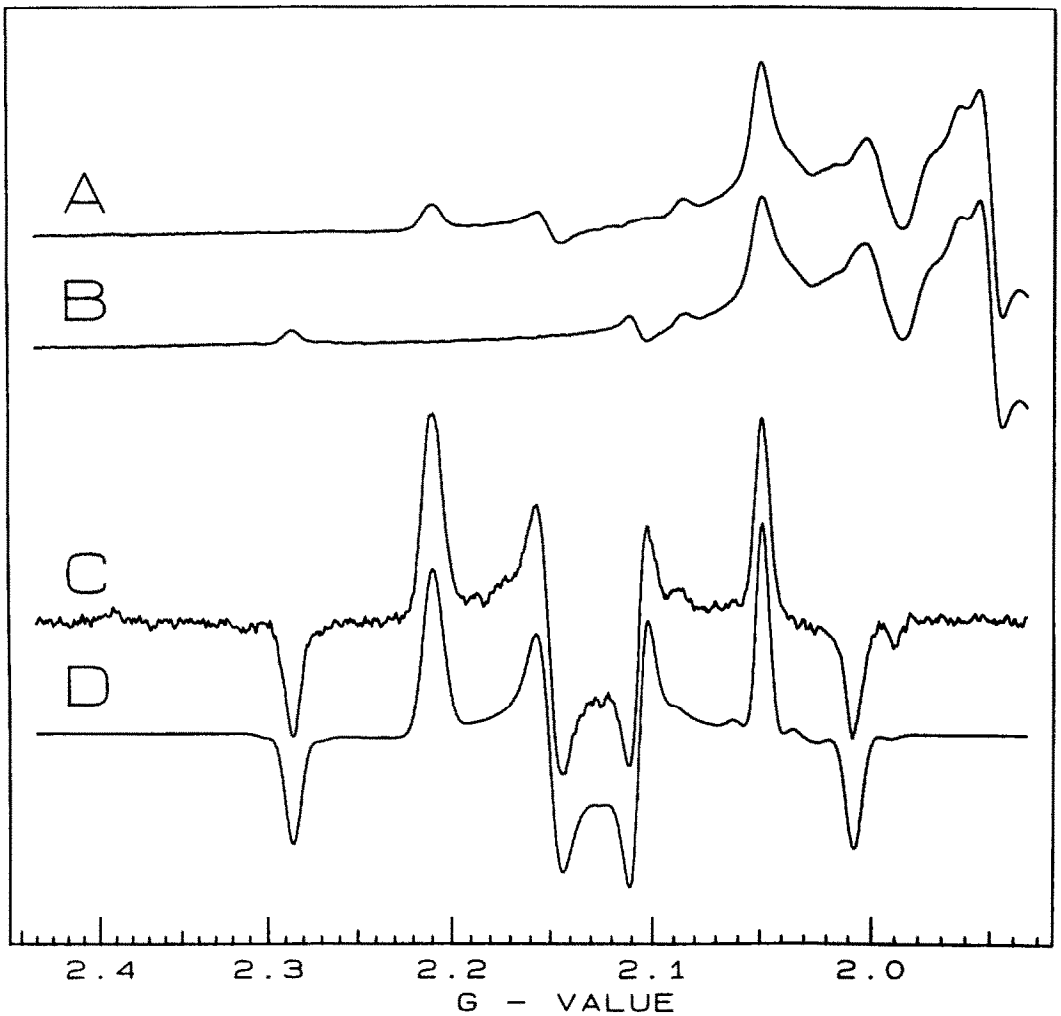

Fig. 1. EPR spectra of the natural selenium-containing F_{420}-non-reducing hydrogenase from *M. voltae*. (A) Hydrogenase as isolated. The sample was dark-adapted prior to the measurement (see section 2). (B) Spectrum after illumination of the sample for 5 min at 35 K. EPR conditions: microwave frequency, 9424.4 MHz; microwave power incident to the cavity, 26 mW; modulation amplitude, 0.638 mT; temperature, 35 K. (C) Difference spectrum of A minus B. (D) Simulation of trace C. This is a summation of 4 different simulations with different weights. Parameters: 'Dark' signal (natural Se): $g_{x,y,z}$ = 2.2094, 2.15045, 2.0088 and widths (x,y,z) = 1.44, 1.4, 1.17 mT; weigth = 1. 'Light' signal (natural Se): $g_{x,y,z}$ = 2.04856, 2.10665, 2.28497 and widths (x,y,z) = 0.954, 1.077, 1.13 mT; weight = −1. 'Dark' signal (^{77}Se): g values and widths as above; $A_{x,y,z}$ = 0.96, 1.548, 5.32 mT; weight = 0.082; 'Light' signal (^{77}Se): g values and widths as above; $A_{x,y,z}$ = 4.327, 4.665, 3.81 mT; weight = −0.082.

A remarkable feature, observed here for the first time, is that the EPR signal of the 'dark' $Ni_a(I) \cdot H_2$ species (Ni-C) shows a highly anisotropic hyperfine splitting due to ^{77}Se. The splitting in the z direction (5.32 mT) demonstrates that the unpaired spin in the d_z2 orbital points directly to the Se nucleus. The much smaller splitting in the x,y directions suggests that orbital overlap of the d_z2 orbital with an sp hybrid of Se also contributes to some extent to the overall splitting.

After illumination of the sample none of the g values is close to 2 anymore (lowest value 2.05). This indicates [33], that the unpaired electron is no longer in an orbital with d_z2 character. Yet, in this 'light' $Ni_a(I)$ state, the ^{77}Se nucleus still has a strong almost isotropic interaction with the unpaired spin. This demonstrates that the unpaired spin has a finite probability at the Se nucleus (via s-character contribution from a Se sp hybrid orbital). It then directly follows that the z direction of the g tensor axis system, which was along the Ni-Se bond in the 'dark' non-perturbed Ni site, is no longer in this direction after photodissociation of the bound hydrogen. Since now an orbital most likely with mainly $d_{x^2-y^2}$ character is pointing to the Se nucleus, it follows that the new z axis is perpendicular to this axis. Thus, the breakage of a bond with bound hydrogen is proposed to result in a 90° flip-over of the electronic z direction. Since the selenium is part of a selenocysteine [9,16] and is therefore in the rigid spatial structure of the polypeptide, and since the light-sensitive changes can also be observed at 4.2 K, a change in the geometric position of the selenium is highly unlikely.

These results call for a re-evaluation of several previous observations.

I. *The effect of H/D on the EPR spectra of the $Ni_a(I)$ state.*

Van der Zwaan et al. [27] have reported that the only effect of exchanging H_2/H_2O by D_2/D_2O on the EPR line

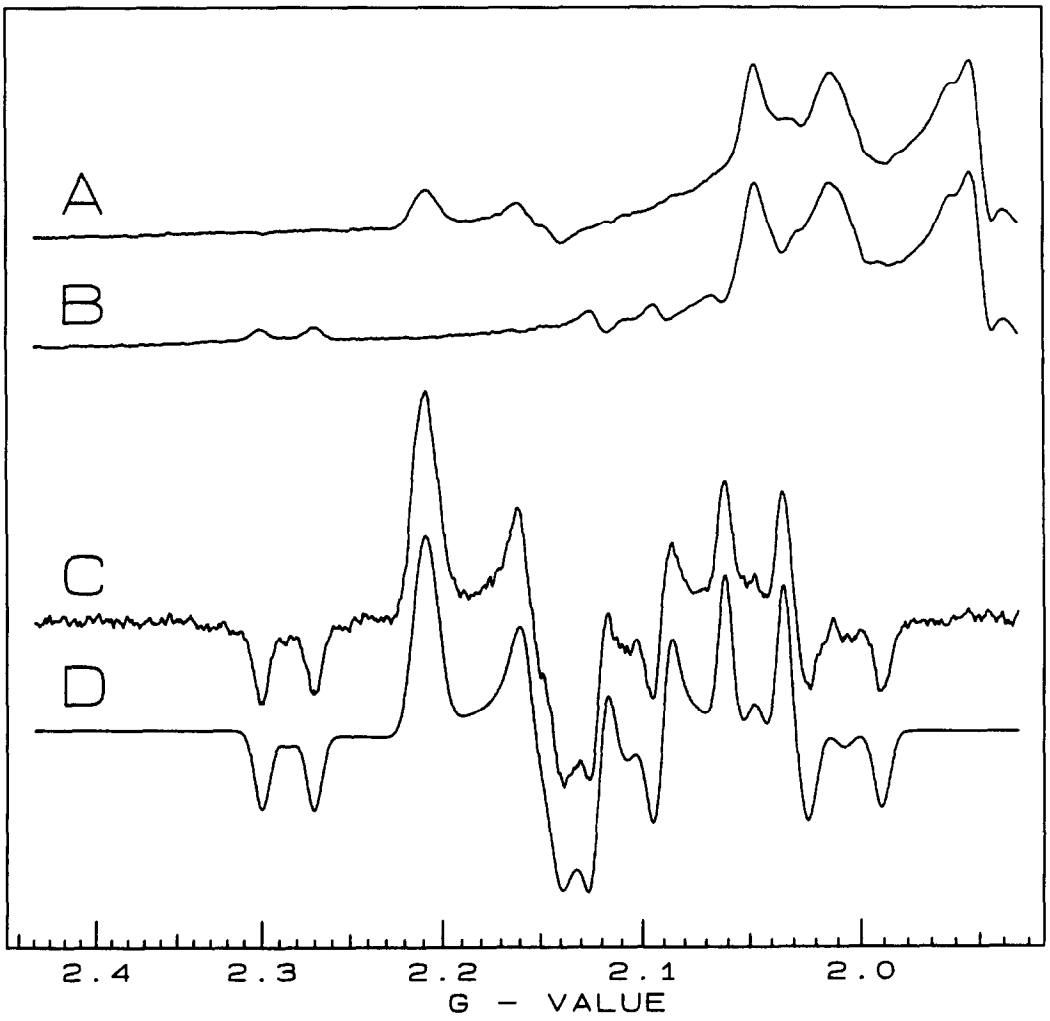

Fig. 2. EPR spectra of the F_{420}-non-reducing hydrogenase from *M. voltae* 92% enriched in ^{77}Se. (A) Hydrogenase as isolated (dark adapted). (B) Spectrum after illumination of the sample for 5 min at 35 K. EPR conditions were as in Fig. 1. (C) Difference spectrum of A minus B. (D) Simulation of trace C. This is a summation of 4 different simulations with different weights. Parameters as in Fig. 1, but for the weights. 'Dark' signal (natural Se): weigth = 0.087; 'light' signal (natural Se): weight = −0.087; 'dark' signal (^{77}Se): weight = 1; 'light' signal (^{77}Se): weight = −1.

shapes of the Ni-C site in *Chromatium vinosum* hydrogenase was a 0.5 mT sharpening of the g_y line of the 'dark' spectrum. The rate of photodissociation slowed down nearly 6 times due to the introduction of deuterium whereas no effect was observed on the light-induced EPR signals. In the *M. voltae* enzyme (Figs.1 and 2) the unpaired spin in the d_z2 orbital of the 'dark' $Ni_a(I)\cdot H_2$ state is pointing directly to the Se nucleus. We have also closely inspected the EPR spectra of this enzyme in D_2O under 5% H_2 (so essentially in a D_2O/D_2 atmosphere due to the inherent H/D exchange activity of nickel hydrogenases). Although we noticed a retarded photodissociation, we could not observe any changes in line widths of the 'dark' $Ni_a(I)\cdot H_2$ signal. Thus, although the unpaired spin in the d_z2 orbital experiences a 5.32 mT, mainly dipole–dipole, interaction of the ^{77}Se nucleus, there was no observable effect of an H/D exchange. These combined findings in the same enzyme definitely eliminate a hydride in a position opposite to the Se, in line with earlier suggestions.

II. *The effect of ^{13}CO on the EPR spectrum of nickel.*

Van der Zwaan et al. [28,29] have demonstrated that CO can replace hydrogen as a ligand to $Ni_a(I)$ in the *C. vinosum* enzyme and that after illumination of either the hydrogen adduct or the CO adduct one and the same EPR spectrum was obtained. Cammack and coworkers have also demonstrated this for the *D. gigas* enzyme [34]. The effect of ^{13}CO was a nearly isotropic splitting of 3.0 mT in the 'dark' $Ni_a(I)\cdot CO$ signal [29]. As one of the g values was close to g_e Van der Zwaan et al. [29] concluded that orbital overlap of the d_z2 orbital with the sp hybrid of the ^{13}C caused this splitting. No splitting was observed after breakage of the bond. This experiment convincingly demonstrated that hydrogen and CO can bind on the same site to nickel. A rather frustrating question, however, remained: why is ^{13}CO causing an isotropic hyperfine splitting of 3.0 mT, whereas the bound hydrogen nucleus (or nuclei) do virtually not show up in the EPR spectra, and, as again demonstrated in this report, has no effect on the width of the g_z line? In model compounds of Ni(I) an axial hydride gives rise to a nearly isotropic hyperfine splitting of 10 mT [35]. As a possible way out it was proposed [29] that dihydrogen might be the ligand. But even then one would intuitively expect some effect of a H/D exchange in the line width of the g_z.

At present we see one possible way to explain all this. The EPR spectra of the ^{77}Se-enriched enzyme presented in this paper demonstrate that the electronic z axis is along the Ni-Se bond in the 'dark' $Ni_a(I)\cdot H_2$ species (Ni-C state). The electronic z axis flips over by 90°, however, after photodissociation of hydrogen and the unpaired spin then is in a, most likely, $d_{x^2-y^2}$ orbital, directly pointing to Se. In view of the absence of any observable effect of an H/D exchange on any of the EPR spectra encountered in this study, we assume as a working hypothesis that hydrogen is bound in a direction perpendicular to the Ni-Se axis. Hence also CO has to bind in the same position. In order to explain the isotropic ^{13}C-hyperfine splitting oberved by Van der Zwaan et al. [29], we have to assume that the electronic z axis, which is along the Ni-Se bond in the 'dark' $Ni_a(I)\cdot H_2$ species, makes a 90° flip upon binding of CO, now pointing directly to the CO. We have depicted these ideas in a model in Fig. 3.

From the present results, we cannot exclude the possibility that the ligand site opposite to the hydrogen/CO binding site is vacant. As indicated by the conservative amino-acid sequence DPCxxC at the carboxy-terminal region in the large subunit of all nickel hydrogenases [5–7,36], EPR studies with ^{33}S-enriched *Wolinella succinogenes* hydrogenase [37] and EXAFS measurements on the enzymes from *D. gigas* [38], *D. baculatus* [14] and *Thiocapsa roseopersicina* [39], it is highly likely that at least one of the other ligands is sulfur. The model can explain the missing H-hyperfine interaction in the g_z line of the 'dark' $Ni_a(I)\cdot H_2$ signal: the unpaired spin is not pointing to the hydrogen. If CO is bound instead of hydrogen, then the flipping electronic z axis explains the

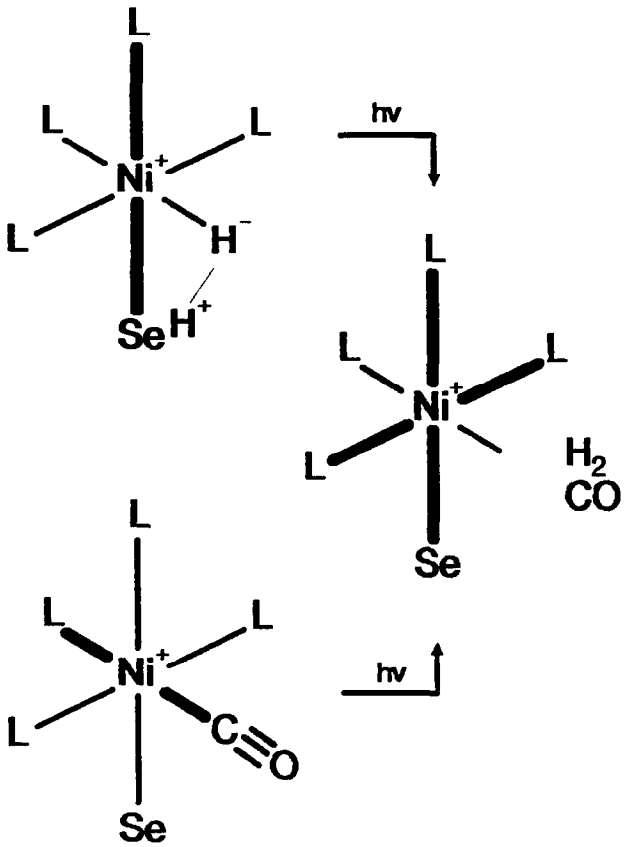

Fig. 3. Working hypothesis for the coordination of nickel in nickel hydrogenases. The thick lines denote either a d_z2 type of orbital or a $d_{x^2-y^2}$ orbital holding the unpaired electron. Beside selenium the other ligands are expected to be oxygen and sulfur, rather than nitrogen.

observed ^{13}C splitting. Photodissociation of either the hydrogen or the CO results in the same coordination. If this model is right then an EPR spectrum of the ^{13}CO adduct of the 'dark' $Ni_a(I)$ state in a ^{77}Se-enriched enzyme should exhibit only hyperfine splitting of the ^{13}C nucleus, but not of the ^{77}Se nucleus. After photodissociation of the CO, however, the 'light' $Ni_a(I)$ spectrum should be the same as shown in Fig. 2, trace B and should show only hyperfine interaction of the ^{77}Se nucleus. This remains to be verified experimentally.

It can be anticipated, that nature had some reason to invoke a selenocysteine in some nickel hydrogenases. The specific hydrogen-uptake activity of the *M. voltae* F_{420}-non-reducing enzyme [9] is 50- to 100-fold greater than the activities found in ordinary nickel hydrogenases. The presence of selenium in the *D. baculatus* enzyme apparently considerably changed the H_2/HD ratio in D_2/H_2O exchange measurements [10,40]. One of the possibilities therefore is that the $R\text{-}Se^-$ ligand acts as a base in order to help in the heterolytic cleavage of H_2. In that case, binding of H^+ to the $R\text{-}Se^-$ group might influence the orbital overlap of sp orbitals of selenium with the d orbitals of nickel. If this is so, then the magnitude of the isotropic splitting of ^{77}Se in the 'dark' $Ni_a(I)\cdot H_2$ signal might be dependent on the pH. This remains to be verified experimentally. In view of the large isotropic interaction of ^{77}Se observed in the 'light' $Ni_a(I)$ signal, photodissociation of the bound hydrogen might also remove the proton bound to selenium.

The model also suggests that it is less likely that any of the ligands is nitrogen, since there is no evidence for N-hyperfine splitting in EPR spectra of the 'dark' $Ni_a(I)\cdot H_2$ species, the 'dark' $Ni_a(I)\cdot CO$ species or the 'light' $Ni_a(I)$ species.

This study demonstrates that the the selenium-containing F_{420}-non-reducing hydrogenase from *M. voltae* is exceptionally well suited to further explore the details of the hydrogen-activating site in nickel hydrogenases.

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