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Published in:
The Journal of Biological Chemistry

Citation for published version (APA):

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Interactions of $^{77}\text{Se}$ and $^{13}\text{CO}$ with Nickel in the Active Site of Active $F_{420}$-nonreducing Hydrogenase from Methanococcus voltae*

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From \‡Molecular Genetics, Department of Biology, University of Marburg, Karl-von-Frisch-Strasse, D-35032 Marburg/Lahn, Germany and \¶E. C. Slater Institute, BioCentrum Amsterdam, Plantage Muidergracht 12, NL-1018 TV Amsterdam, The Netherlands

The selenium-containing $F_{420}$-nonreducing hydrogenase from Methanococcus voltae was prepared in the Ni$_a$(I)-CO state. The effect of illumination on this light-sensitive species was studied. EPR studies were carried out with enzyme containing natural selenium or with enzyme enriched in $^{77}\text{Se}$. Samples were prepared with either CO or $^{13}\text{CO}$. In the Ni$_a$(I)-CO state, the nuclear spins of both $^{77}\text{Se}$ ($I = 1/2$) and $^{13}\text{C}$ ($I = 1/2$) interacted with the nickel-based unpaired electron, suggesting that they are positioned on opposite sites of the nickel ion. In the light-induced signal, the interaction with $^{13}\text{CO}$ was lost. The $^{77}\text{Se}$ nuclear spin introduced an anisotropic hyperfine splitting in both the dark and light-induced EPR signals. The data on the active enzyme of $M. \text{voltae}$ are difficult to reconcile with the crystal structure of the inactive hydrogenase of Desulfovibrio gigas (Volbeda, A., Charon, M. H., Piras, C., Hatchikian, E. C., Frey, M., and Fontecilla Camps, J. C. (1995) Nature 375, 580–587) and suggest a structural change in the active site upon activation of the enzyme.

Hydrogenases catalyze the heterolytic cleavage of molecular hydrogen into a hydride and a proton (1, 2), and the oxidation of the hydride to a proton and two electrons. The protons are released, and the electrons are transferred to an electron acceptor. Hydrogenases also catalyze the reverse reaction. All hydrogenases, which can activate hydrogen in the absence of added cofactors, contain transition metals as essential components. On the basis of their amino acid sequences and metal contents, hydrogenases can be divided into two classes. One class consists of enzymes containing only iron. These enzymes are called Fe-hydrogenases or Fe-only hydrogenases (3). The Fe-hydrogenases contain at least two classical (4Fe-4S) clusters (4, 5), in addition to a special cluster involving 4–7 Fe atoms (6), originally proposed to be a novel 6Fe cluster forming the hydrogen-activating site (H-cluster) (7, 8).

The second class comprises hydrogenases containing nickel in addition to iron. These enzymes are called Ni-hydrogenases (also Ni-Fe or [NiFe] hydrogenases). The nickel ion is essential for activity (9, 10). It responds to changes in the redox potential (11–13) and is generally believed to be the heart of the active site (for review, see Ref. 14). Some Ni-hydrogenases contain selenium in the form of selenocysteine (15–17) and are often called [NiFeSe] hydrogenases. They were earlier considered to be a subclass of the Ni-hydrogenases. Comparison of the amino acid sequences (18), however, shows that the basic unit of all known Ni-hydrogenases is the same (14) and the only difference in the Se-containing enzymes is a replacement of a conservative Cys residue by a Sec residue (17).

Depending on the redox potential, the nickel ion can have an unpaired electron. Consequently, nickel hydrogenases have been intensively studied by EPR spectroscopy. Most hydrogenases, when aerobically purified, show two distinct EPR signals due to Ni(III), often within the same preparation. The major difference in the EPR signals is the position of the $g_\parallel$ line of the rhombic signal: it can be either at $g = 2.24$ or at $g = 2.16$ (the $g_\parallel$ lines around 2.3 and the $g_\perp$ lines around 2.0 differ only slightly). Fernandez et al. (19) discovered that enzyme preparations showing a Ni(III) signal with a $g_\parallel$ value of 2.16 were activated by hydrogen within a few minutes, but that several hours were required to activate enzyme molecules with Ni(III) showing a $g_\parallel$ line at 2.24. The states were termed termed “ready” and “unready,” respectively. Complete reduction resulted in an active enzyme. In this paper, nickel in ready and unready enzyme will be referred to as Ni$_a$ and Ni$_b$, respectively. In the literature these states have been called Ni-a and Ni-b (20) or Ni-B and Ni-A (21), respectively. Nickel in active enzyme is called Ni$_{act}$. It must be remembered that both the ready and unready enzymes are still inactive and need a reductive activation.

During reductive redox titrations, the $S = 1/2$ EPR signals of Ni(III) disappear and EPR-silent intermediates are obtained (22), in which the nickel ion is considered to be in the divalent, diamagnetic state (14). At temperatures below 10 °C the enzyme remains inactive (23). Only after a reductive treatment at elevated temperatures can the enzyme become active, and only then is it possible to further reduce the divalent nickel ion. This leads to another $S = 1/2$ EPR signal with $g$ values of 2.19, 2.14, and 2.02 (13, 19, 21, 22, 24, 25). As this signal was the third EPR signal from nickel in hydrogenases, it is often called Ni-C. It corresponds to an active state of the enzyme. In the Se-containing enzyme from Desulfovibrio baculatus, this state has slightly different $g$ values (2.23, 2.17, and 2.01) (26). This signal is maximal in enzyme under 1% H$_2$ (27) and then represents about 55% (pH 8–9) to 90% (pH 6) of the nickel concentration. Complete reduction under 200 kPa of H$_2$ leads to a loss of this EPR signal.

Van der Zwaan et al. (25) discovered that the species responsible for this signal in the Chromatium vinosum enzyme is light-sensitive at temperatures below 77 K and proposed that this photodissociation involves the breakage of a bond between monovalent nickel and some form of hydrogen. The correspond-

* This work was supported by the Deutsche Forschungsgemeinschaft and Fond der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Sec, selenocysteine; mT, millitesla.
ing state of nickel will be denoted here as Niₐ(I)-H₂ (or Ni-C). Illumination of this state with white light resulted in an EPR spectrum with the lowest g value at 2.05. When performed in D₂O, the rate of photodissociation was nearly 6 times slower and the EPR lines before illumination were slightly sharper (maximally 0.5 mT) than in H₂O. After photodissociation the EPR lines were equally sharp in H₂O and D₂O. In this report the photoproduction of the Ni₁(I)-H₂ state will be referred to as Ni₁(I)-[H₂].

It has been reported that treatment of Ni-hydrogenases in the most reduced state (under 200 kPa of H₂, here called the N₀ state) with the competitive inhibitor CO leads to yet another EPR signal (28, 29). The CO-induced species Ni₁(I)-CO was also light-sensitive (28). Illumination resulted in an EPR signal virtually identical to that obtained after illumination of the Ni₁(I)-H₂ state. Hence it was ascribed to a Ni₁(I)[CO] state. Indeed, when prepared with ¹³CO, a nearly isotropic 2-fold splitting of the EPR signal was obtained for the I = 1/2 spin of the ¹³C nucleus (30). No effect of ¹³C on the spectrum was observed after illumination. Likewise, no effects of replacement of H₂O by D₂O, neither on the line widths nor on the rate of photodissociation, could be observed in this case. The authors concluded that hydrogen and CO can apparently bind to nickel at the same position, resulting in different EPR spectra in the dark, but identical spectra after photodissociation. As the EPR of the Ni₁(I)[¹³CO] species clearly showed an interaction between the unpaired spin of the nickel and the ¹³C nucleus (30), the question arose as to why bound hydrogen only gave a slight line broadening, although the proton is a much stronger magnetic field than ¹³C.

More recently, we have reported (31) that the anaerobically purified selenium-containing F₄₃₀-nonreducing hydrogenase from M. voltae also displays a light-sensitive EPR signal with all the characteristics of the Ni₁(I)-H₂ state (unpaired electron in an orbital with predominantly dₓ² character). With ⁷⁷Se-enriched enzyme, an anisotropic 2-fold splitting due to the I = 1/2 nuclear magnetic moment of the isotope was observed, which was 5.3 mT in the z direction, but much smaller (about 0.96 and 1.49 mT) in the x and y directions. After photodissociation of bound hydrogen, the resulting EPR signal (lowest g value at 2.05, so unpaired electron no longer in dₓ² orbital) showed a nearly isotropic splitting due to ⁷⁷Se. A reasonable way to explain these data was to assume a 90° flip of the electronic z-axis upon photodissociation. No effects on the line widths could be observed when experiments were performed in D₂O. This means that in the Ni₁(I)-H₂ state the unpaired spin in a dₓ² orbital is pointing to Se, but not to hydrogen. Consequently, it was concluded that hydrogen could be bound perpendicular to the Ni-Se axis. Since it is assumed that CO and hydrogen bind to the same site (30), such a proposal can be checked by studying the Ni₁(I)-¹³CO state in the Se-enzyme.

In this contribution we describe the EPR properties of CO-treated selenium-containing F₄₃₀-nonreducing hydrogenase from M. voltae. We have used normal or ⁷⁷Se-enriched enzyme treated with normal CO or ¹³CO to further investigate the interactions of the unpaired electron of the nickel center. In addition, we have investigated whether there is a pH effect on the anisotropy of the splitting caused by ⁷⁷Se in the Ni₁(I)-H₂ species. The results are discussed in light of the recent finding that the active site of [NiFe] hydrogenases is a bimetallic cluster (lines below 2.05).
Interactions of $^{77}\text{Se}$ and $^{13}\text{CO}$ with Nickel in Hydrogenase

**The Nia(I)$[\text{H}_2]$ and Nia(I)$[\text{H}_2]$ States**

EPR spectra of $^{77}\text{Se}$-enriched Se-containing hydrogenases have shown that this element is a ligand to nickel (26, 31). The observed interactions of the nickel-based unpaired electron with the $^{77}\text{Se}$ nuclear magnetic moment (31) opened the possibility to use the Ni-Se axis as an internal reference frame. This already led to the conclusion (31) that in the Nia(I)$[\text{H}_2]$ state, the bound hydrogen cannot be present as a ligand to nickel in a position opposite to Se, since spectra recorded with a sample in D$_2$O were not distinguishable from those prepared in H$_2$O. As this is an important observation in the frame of the discussion in this paper, we show the spectra in Fig. 2.

Carbon monoxide enriched in $^{13}\text{C}$ had a prominent effect on the EPR spectrum of the Nia(I)$[\text{CO}]$ species in the C. vinosum enzyme (30), showing that the electronic $z$-axis was along the Ni-CO bond. As outlined in the Introduction, it was assumed that H$_2$ and CO could bind to the same position. This raised the question why bound $^{13}\text{CO}$ caused a strong hyperfine splitting, whereas bound hydrogen had only a minor effect. To further address this problem, we have investigated the binding of CO and $^{13}\text{CO}$ to active Se-containing M. voltae enzyme with and without $^{77}\text{Se}$. Both $^{77}\text{Se}$ and $^{13}\text{C}$ have a nuclear spin of $1/2$ and can introduce a 2-fold splitting of the nickel EPR signals if the free electron is interacting with the magnetic ligand. This effect is expected to be considerable if the orbital containing the unpaired spin is pointing toward the ligands.

**The Nia(I)$[^{13}\text{CO}]$ and Nia(I)$[^{13}\text{CO}]$ States**

Carbon monoxide enriched in $^{13}\text{C}$ had a prominent effect on the EPR spectrum of the Nia(I)$[\text{CO}]$ species in the C. vinosum enzyme (30), showing that the electronic $z$-axis was along the Ni-CO bond. As outlined in the Introduction, it was assumed that H$_2$ and CO could bind to the same position. This raised the question why bound $^{13}\text{CO}$ caused a strong hyperfine splitting, whereas bound hydrogen had only a minor effect. To further address this problem, we have investigated the binding of CO and $^{13}\text{CO}$ to active Se-containing M. voltae enzyme with and without $^{77}\text{Se}$. Both $^{77}\text{Se}$ and $^{13}\text{C}$ have a nuclear spin of $1/2$ and can introduce a 2-fold splitting of the nickel EPR signals if the free electron is interacting with the magnetic ligand. This effect is expected to be considerable if the orbital containing the unpaired spin is pointing toward the ligands.

**Fig. 2** Comparison of difference spectra (Nia(I)$[\text{H}_2]$ minus Nia(I)$[\text{H}_2]$) of samples prepared in H$_2$O and D$_2$O. A, difference spectrum of a sample dissolved in H$_2$O. B, difference spectrum of a sample dissolved in D$_2$O. EPR conditions: microwave frequency, 9424.4 MHz; microwave power, 26 milliwatts; modulation amplitude, 0.638 mT; temperature, 35 K.

Fig. 3 displays the spectra obtained with a sample treated with $^{13}\text{CO}$. In trace A, the dark (Nia(I)$[^{13}\text{CO}]$) spectrum is shown. Comparison with Fig. 1A shows that the $g_{xy}$ line at $g = 2.11$ apparently undergoes a 2-fold splitting (2.0 mT) caused by the introduction of the $^{13}\text{C}$ nuclear spin. This points to an interaction between the nuclear spin of the $^{13}\text{C}$ nucleus and the unpaired electron of the nickel. Trace B shows a spectrum recorded after illumination of the same sample in the cavity. Although this light (Nia(I)$[^{13}\text{CO}]$) signal is rather anisotropic, one can detect the low-field features around $g = 2.33$ and the high-field line at $g = 2.05$. These lines show neither hyperfine splitting nor line broadening (compare Fig. 1B). This indicates that after illumination there is no longer an interaction between the unpaired electron of the nickel and the nuclear spin of the $^{13}\text{C}$, in line with the assumption that illumination leads...
to photolysis of the Ni-CO bond. Trace C displays the difference spectrum dark minus light. Comparison with Fig. 1 indicates that the $g_z$ line of the Ni\(_a\)(I)\([13CO]\) signal around $g = 2.01$ is split by $^{13}$C as well. This region of the spectrum might be somewhat less reliable because the sharp feature in the dark and light spectra can cause some artifacts in the difference spectrum. It can also be seen that the highest $g$ value of the Ni\(_a\)(I)\([CO]\) state is at 2.33, whereas the highest $g$ value of the Ni\(_a\)(I)\([H_2]\) state (Fig. 1D) is at 2.285. This is unlike the situation observed in the C. vinosum enzyme (25, 30).

The Effect of $^{77}$Se Enrichment

A sample enriched in $^{77}$Se and treated with CO yielded a spectrum as shown in Fig. 4. Trace A displays the difference spectrum dark minus light. Comparison with Fig. 1E indicates that the $g_x$ line of the Ni\(_a\)(I)\([13CO]\) signal around $g = 2.01$ is split by $^{13}$C as well. This region of the spectrum might be somewhat less reliable because the sharp feature in the dark and light spectra can cause some artifacts in the difference spectrum. It can also be seen that the highest $g$ value of the Ni\(_a\)(I)\([CO]\) state is at 2.33, whereas the highest $g$ value of the Ni\(_a\)(I)\([H_2]\) state (Fig. 1D) is at 2.285. This is unlike the situation observed in the C. vinosum enzyme (25, 30).

**Effect of pH**

As mentioned in the Introduction, an anisotropic splitting due to $^{77}$Se was observed in $^{77}$Se-enriched samples in the Ni\(_a\)(I)\([H_2]\) state. This could have been an effect of partial protonation of the selenol group. It has been speculated that this group might serve as a basic group, which could bind the proton originating from the heterolytic cleavage of $H_2$. In this case the shape of the spectrum might be expected to change with changing pH. This notion was tested by taking spectra of $^{77}$Se-enriched samples in the Ni\(_a\)(I)\([H_2]\) state in the range of pH 6 to 9 (Fig. 5). In this range no changes in the spectra were observed.

**Computer Simulation**

The data in Figs. 1, 3, and 4 show that in the dark state the unpaired electron of nickel interacts with both the $^{77}$Se nucleus and the $^{13}$C nucleus of CO. After illumination interaction with the $^{13}$C nucleus was lost. In contrast, the $^{77}$Se nucleus still interacted with the unpaired spin after illumination.

To verify this interpretation and to more precisely determine the $g$ values and hyperfine tensors, simulations of the experimental spectra were made. Where necessary, the contributions to the spectra of the Ni\(_a\)(I)\([H_2]\) and Ni\(_a\)(I)\([H_2]\) species were

Fig. 3. EPR spectra of the Ni\(_a\)(I)\([13CO]\) state of the M. voltae hydrogenase in the dark and after illumination. A, the Ni\(_a\)(I)\([13CO]\) form in the dark. B, the Ni\(_a\)(I)[13CO] form as obtained after 5-min illumination at 70 K with white light. The illumination was continued during the measurement. C, difference spectrum of A minus B. For the EPR conditions, see Fig. 1.

Fig. 4. EPR spectra of the Ni\(_a\)(I)\([CO]\) state of $^{77}$Se-enriched M. voltae hydrogenase in the dark and after illumination. A, the Ni\(_a\)(I)\([CO]\) form in the dark. B, the Ni\(_a\)(I)[CO] form as obtained after 5 min illumination at 70 K with white light. The illumination was continued during the measurement. The arrows point to the split high-field line. C, difference spectrum of A minus B. The arrow indicates the position were a member of the split $g_x$ line of the Ni\(_a\)(I)\([CO]\) can be detected. For the EPR conditions, see Fig. 1.
subtracted. Dark minus light difference spectra as in Fig. 1
were used for this purpose. In view of the limited quality of the EPR spectra, resulting from
the limited amounts of isotope-enriched enzyme and overlapping Fe-S, radical, and other background signals (Figs. 1, 3,
and 4), two different approaches for computer simulations were
applied.

Approach I—In this approach, the line shapes of the dark
minus light differencespectra were obtained after conversion
of all spectra to the same microwave frequency (9420 MHz). It
was assumed that features below
\( g = 2.03 \) in the difference
spectra were not very reliable due to sharp lines in the parent
spectra. Simulation were performed with the program of F.
Neese (35).

Comparisons of experimental and simulated spectra are shown in Fig. 6. The Ni\( _{\text{x}} \)(I)−CO signal was best simulated as the sum of two axial signals with slightly different \( g \) values, but
with considerably different \( W \) values. In addition, the Ni\( _{\text{x}} \)
(I)(CO) spectrum was simulated as a sum of two different rhombic signals. For such simulation, the enrichment in \( ^{77}\text{Se} \) was taken into account (7.58% for normal samples and 92% for the
\( ^{77}\text{Se} \)-enriched samples). Hence, every simulated difference
spectrum consisted of a sum of eight signals. The EPR parameters of these eight signals are summarized in Table I, and the relative intensities, used to compose the spectra in Fig. 6 (trac-
eses B, D, and F) are given in Table II. Note that the ratio of the
two dark signals equals the ratio of the two light-induced signals. Using this approach, it thus seems that two slightly
different species of the Ni\( _{\text{x}} \)(I)−CO form were present. It was

<table>
<thead>
<tr>
<th>Signal</th>
<th>( g ) values</th>
<th>Line width (mT)</th>
<th>Hyperfine splitting (mT)</th>
</tr>
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<tr>
<td></td>
<td>( g_x )</td>
<td>( g_y )</td>
<td>( g_z )</td>
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<tr>
<td>Dark 1 CO/Se</td>
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<td>2.1094</td>
<td>2.0123</td>
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<td>1.75</td>
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<tr>
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<td>2.26</td>
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<td>2.1090</td>
<td>2.3200</td>
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<tr>
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<td>7.39</td>
<td>0.95</td>
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<tr>
<td>Light 2 CO/Se</td>
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<td>2.1303</td>
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<tr>
<td>Light 2 ( ^{77}\text{Se/CO} )</td>
<td>3.49</td>
<td>1.30</td>
<td>1.01</td>
</tr>
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</table>

**Fig. 5.** Comparison of the \( ^{77}\text{Se} \) hyperfine splitting in EPR sig-
nals of samples in the Ni\( _{\text{x}} \)(I)−H\(_2\) state at different pH values.
Displayed are difference spectra of Ni\( _{\text{x}} \)(I)−H\(_2\) minus Ni\( _{\text{x}} \)(I)\([\text{H}_2]\). A, sam-
ple at pH 6. B, sample at pH 7.5. C, sample at pH 9. For EPR conditions
see Fig. 2.

**Fig. 6.** Comparison of the difference spectra from Figs. 1–3
with the corresponding simulated spectra using simulation ap-
proach I. A, Ni\( _{\text{x}} \)(I)−CO minus Ni\( _{\text{x}} \)(I)(CO) (same as Fig. 1E).
B, simulation of A. C, Ni\( _{\text{x}} \)(I)−\( ^{13}\text{CO} \) minus Ni\( _{\text{x}} \)(I)(\( ^{13}\text{CO} \)) (same as Fig. 3C). D,
simulation of C. E, as in A but prepared from \( ^{77}\text{Se} \)-enriched hydrogen-
ase (same as Fig. 4C). The parameters and relative intensities used for
the simulations are shown in Tables I and II, respectively.
noted that the ratio of these forms was constant in all simulations of spectra of several different samples. As can be seen in Fig. 6, the simulated spectra fitted quite well in the region above \( g \) = 2.03, but below that value too much intensity was obtained for the \( g \) line of the Ni\(_a\)(I)-CO signal.

**Approach II**—Here it was assumed that, like in our previous studies on the Ni\(_a\)(I)H\(_2\) state (31), the region below \( g \) = 2.03 was also a reliable representation of the EPR line shapes of the Ni\(_a\)(I)-CO and Ni\(_a\)(I)[CO] states. For an overall comparison, slight differences in experimental microwave frequency among the EPR tube were not corrected. Illumination of a sample did not change this frequency. The programs of S. P. J. Albracht (36) were used.

The Ni\(_a\)(I)-CO spectrum was simulated as a single axial signal, whereas the spectrum obtained after illumination, the Ni\(_a\)(I)[CO] state, was an overlap of two signals with different \( g \) (and \( W \)) values around \( g = 2.33 \), but the same \( g \), and \( g \) values and widths. Fig. 7 summarizes the results (see also Tables III and IV). Now the features below \( g \) = 2.03 fitted reasonably well.

**DISCUSSION**

**Conclusions from the Simulations**—Simulations according to approach I gave quite good fits, except for the region below \( g \) = 2.03. Following approach II, much better fits were obtained for this particular region, but now the low-field shoulder around \( g = 2.13 \) in Fig. 7A could not be accounted for. In using approach II, it was noticed that all simulations shown in Fig. 7 differed in the \( g = 2.13 \) region in the same way from the experimental dark minus light spectra of the three different samples, and hence this difference was not sensitive to \(^{13}\text{CO}\) or \(^{77}\text{Se}\). It is presumably due to a light-sensitive change not related to the nickel site. It was this feature that determined whether the spectrum of the Ni\(_a\)(I)-CO state was simulated as one species (approach II) or two species (approach I). In view of the better overall fits with approach II, we conclude that there is presumably only one Ni\(_a\)(I)-CO state.

In evaluating both approaches, we come to the following conclusions about the effects of CO, \(^{13}\text{CO}\), and \(^{77}\text{Se}\) on the EPR spectra. Like in the C. vinosum and D. gigas enzymes, CO binds to the nickel site in the M. voltae enzyme in a light-sensitive way. The Ni\(_a\)(I)-CO state is presumably homogeneous as in the other enzymes, whereas in the Ni\(_a\)(I)[CO] state two major components can be detected by EPR. Unlike the situation in the C. vinosum hydrogenase, the Ni\(_a\)(I)[CO] state has an EPR spectrum differing from that of the Ni\(_a\)(I)H\(_2\) state. Reaction of the enzyme with \(^{13}\text{CO}\) results in a nearly isotropic interaction \( A_{\text{gy}} = 2.2, 2.2, \) and 2.35 mT) in the Ni\(_a\)(I)[CO] state. Enrichment with \(^{77}\text{Se}\) gives rise to \( A_{\text{gy}} = 4.6, 4.6, \) and 12.1 mT in the Ni\(_a\)(I)-CO state. After illumination the \( g \) region of the resulting Ni\(_a\)(I)[CO] signals is indistinguishable from that obtained with the \(^{13}\text{CO}\)-treated enzyme. This means that, under the conditions used, the \(^{77}\text{Se}\) nucleus has no detectable hyperfine splitting in this direction. The \( g \) region shows a clear splitting of 3.81 mT. Information on the \( g \) and \( A \) values of the \( g \) region is unreliable due to overlapping background signals and overlap with the \( g \) region of the Ni\(_a\)(I)-CO signal. We have assumed common \( g \) (2.114), \( W \) (2.7 mT), and \( A \) values (3.81

**TABLE II**

<table>
<thead>
<tr>
<th>Trace</th>
<th>Dark 1</th>
<th>Dark 2</th>
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<tbody>
<tr>
<td></td>
<td>CO(_a)</td>
<td>CO(_a)</td>
</tr>
<tr>
<td>B</td>
<td>58.81</td>
<td>4.82</td>
</tr>
<tr>
<td>D</td>
<td>5.09</td>
<td>58.55</td>
</tr>
<tr>
<td>F</td>
<td>-5.09</td>
<td>-58.55</td>
</tr>
</tbody>
</table>

**Fig. 7.** Comparison of the difference spectra from Figs. 1–3 with the corresponding simulated spectra using simulation approach II. A, Ni\(_a\)(I)-CO minus Ni\(_a\)(I)[CO] (same as Fig. 1E). B, simulation of A. C, Ni\(_a\)(I)[CO] minus Ni\(_a\)(I)[13CO] (same as Fig. 3C). D, simulation of C. E, as in A but prepared from \(^{77}\text{Se}\)-enriched hydrogenase (same as Fig. 4C). The original microwave frequencies of the experimental spectra were used (9420–9421.7 MHz). The \( g \) scale is given for 9421 MHz. The parameters and relative intensities used for the simulations are shown in Tables III and IV, respectively.
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The table gives the $g$ values, line widths, and hyperfine-splitting values used for simulation of the different signals. The line width (W) in mT is defined as the peak-to-trough width of the derivative Gaussians used for the simulations; for absorption-type lines this is the width at the inflection points, and for the derivative-type lines this is the peak-to-trough width.

Table III

<table>
<thead>
<tr>
<th>Signal</th>
<th>$g$ values</th>
<th>Line width (mT)</th>
<th>Hyperfine splitting (mT)</th>
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<tbody>
<tr>
<td></td>
<td>$g_x$</td>
<td>$g_y$</td>
<td>$g_z$</td>
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<tr>
<td>Dark</td>
<td>2.1084</td>
<td>2.1084</td>
<td>2.0123</td>
</tr>
<tr>
<td>$^{13}$CO/CO-Se</td>
<td>2.0484</td>
<td>2.1137</td>
<td>2.3200</td>
</tr>
<tr>
<td>Light 1</td>
<td>$^{77}$Se</td>
<td>4.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Light 2</td>
<td>$^{13}$CO/CO-Se</td>
<td>2.0484</td>
<td>2.1137</td>
</tr>
</tbody>
</table>

The EPR parameters are in Table III. As can be seen the natural abundance of $^{13}$C (1.1%) was neglected.

A plausible explanation for the $^{77}$Se- hyperfine splitting in all of these states is that the $z$-axis in both dark states (electron in $d_{z^2}$) is along the Ni-Se axis, but perpendicular to this axis in the light-induced states (electron in $d_{x^2}$). The differences in $^{77}$Se-hyperfine interaction when CO is present points to a difference in orbital overlap between nickel and selenium, probably due to a slightly distorted structure, in line with the difference in $g$ values of the Ni$_i$(I)[H$_2$] and Ni$_i$(I)[CO] states.

Comparison of CO Binding and Hydrogen Binding—$F_{205}$-nonreducing hydrogenase from M. voltae, as anaerobically purified in a glove box containing 5% H$_2$, shows EPR characteristics that are typical for [NiFe] hydrogenases in the Ni$_i$(I)[H$_2$] state (31) (Fig. 1D). Treatment of fully reduced samples with 10% CO resulted in different EPR spectra (Fig. 1A). The change of the $g$ values upon CO treatment indicates binding of CO to nickel, and this is confirmed by the use of $^{13}$CO. We note that the $^{13}$C splittings are somewhat smaller than those observed in the Ni$_i$(I)[H$_2$] state of the C. vinosum hydrogenase (A$_{xy}$ = 2.88, 3.04, and 3.2 mT; Ref. 30). The C. vinosum enzyme showed a rhombic EPR signal ($g_{xy}$ = 2.12, 2.07, and 2.02), whereas the signal of the Ni$_i$(I)[CO] state from the M. voltae enzyme is axial ($g_{xy}$ = 2.11, 2.11, and 2.01). We also note that the $^{77}$Se-hyperfine interaction in the Ni$_i$(I)-CO state (A$_{zy}$ = 4.6, 4.6, and 12.1 mT) is considerably stronger than in the Ni$_i$(I)[H$_2$] state (A$_{xy}$ = 0.96, 1.548, and 5.32 mT) (31).

Illumination at low temperatures of CO-treated M. voltae samples led to a spectrum that differed noticeably from that of the Ni$_i$(I)[H$_2$] state. The highest $g$ value of the former is 2.32 (species 1) to 2.3315 (species 2), whereas the latter has a highest $g$ value of 2.285 (Fig. 1). In this respect the enzymes from M. voltae and C. vinosum also differ; with the C. vinosum enzyme, the $g$ values of the Ni$_i$(I)[H$_2$] and Ni$_i$(I)[CO] states were identical. This suggests that the changes in the conformation of the nickel site induced by binding of the H-species (the Ni$_i$(I)[H$_2$] state) or CO are different in the M. voltae enzyme; after photolysis of the added ligands, the EPR spectra differ. In the C. vinosum enzyme, the H-species and CO apparently induce the same (if any) conformational changes.

There is a second major difference in EPR properties of the Ni$_i$(I)[H$_2$] and Ni$_i$(I)[CO] states in the M. voltae enzyme. In the former state the $^{77}$Se-hyperfine interaction is quite isotropic (A$_{xy}$ = 4.327, 4.665, and 3.81 mT) (31), but in the latter state a stronger anisotropic interaction is observed (A$_{xy}$ = 3.81, 3.81, and 0 mT). This strengthens the notion that CO binding to the M. voltae enzyme perturbs the coordination of the active site more than hydrogen binding.
Based on the assumption that the unpaired electron in the Ni(I)H₂ and Ni(I)CO state is in an orbital with mainly a d₂ character, the results on the Se-containing M. voltae enzyme (this paper and Ref. 31) allow the conclusion that in the Ni(I)H₂ state there is no hydrogen species bound opposite to selenocysteine, but that the site is empty and available for binding of CO or H₂ (27). This is in agreement with the very small effect of H/D exchange on the line width of the Ni(I)H₂ spectra from various hydrogenases (25, 38) including the F₄₂₀ nonreducing enzyme from M. voltae (Fig. 2). It means that the light-sensitive hydrogen species bound to the active site (25, 39, 40) is bound elsewhere. One possibility is binding perpendicular to the z-axis as suggested by Marganian et al. (41, 42) on the basis of Ni(I) model compounds that had reacted with CO or H₂. Another possibility is binding to Fe in the active [NiFe] site. The proton generated upon cleavage of dihydrogen might bind to another ligand.

The protongenerated upon cleavage of dihydrogen to H₂ and Nia(I)H₂ state? In this respect we note that we have never seen to be tested.

In summary, the results presented in this paper suggest that CO can bind to nickel opposite the selenium atom. There is a large amount of delocalization of the electron density of the nickel-based unpaired spin toward both ligands. In the absence of CO the site opposite selenium is vacant. Our data also suggest that the hydrogen species present in the Ni(I)H₂ state might also be present in the Ni(I)CO state. This hydrogen species could be bound perpendicular to the electronic z-axis or to the iron atom. The results in Fig. 5. make a protonation of selenium in the Ni(I)H₂ state in the range of pH 6 to 9 unlikely, since there is no change in g values or hyperfine interaction. The proton generated upon cleavage of dihydrogen might bind to another ligand.

The present results on an active hydrogenase are difficult to reconcile with the x-ray structure of the oxidized, inactive D. gigas enzyme (33). Here the position opposite the sulfur atom, which is analogous to the selenium atom in the M. voltae enzyme, is occupied by another sulfur atom. A different structure in active enzyme is in line with the great change in properties of the active site in nickel hydrogenases upon activation/deactivation (14). No x-ray data are available on the active D. gigas enzyme yet.

Acknowledgments—S. P. J. A. is indebted to the Netherlands Foundation for Chemical Research, for grants, supplied via the Netherlands Organization for Scientific Research, which enabled the purchase of the Bruker ECS 106 EPR spectrometer.