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A new form of the active-site nickel in the [NiFe] hydrogenase from *Allochromatium vinosum*

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A new form of the A. vinosum [NiFe] hydrogenase from *Allochromatium vinosum* was obtained. The EPR spectrum of the oxidized state of this form was very similar to the Ni$\text{L}^*$ state, but this state was not light sensitive, showed no activity and was stable in air. Reduction of this state with dithionite yielded a second state with an EPR spectrum resembling that of enzyme in the Ni$\text{L}^*$ state. The consequences of these results for the model with a bridging hydride coordinated to nickel in the Ni$\text{C}^*$ state, are discussed. Furthermore, an alternative reaction catalysed by the [NiFe] hydrogenase from *A. vinosum* is hypothesised, namely the oxidation of CO to CO$_2$. 
Hydrogenases catalyse the reversible oxidation of hydrogen into two protons and two electrons. The membrane-bound [NiFe] hydrogenase (MBH) of *Allochromatium vinosum* belongs to the standard hydrogenases, just like the one from *Desulfovibrio gigas*. The [NiFe] hydrogenase of this latter bacterium has been crystallised and the structure was refined to a resolution of 2.54 Å (Volbeda, 1995, 1996a). It shows an unprecedented, bimetallic active site, containing a nickel and an iron atom, located in the large subunit (62 kDa) of the heterodimeric enzyme. The Ni and the Fe ion are bridged by two cystein thiol s and the iron atom binds three non-protein ligands that were characterised in the *A. vinosum* enzyme by infrared spectroscopy as one CO and two CN’ ligands (Happe, 1997; Pierik, 1999). The stretching frequencies of these diatomic ligands vary with the different redox states of the enzyme (Bagley, 1995).

The small subunit (32 kDa) harbours three Fe-S clusters in an almost linear arrangement: two [4Fe-4S]^{+/-+} clusters (E_m ~ -400 and -350 mV at pH 8, Teixeira, 1989) with in between a [3Fe-4S]^{1+/0} cluster (E_m ~ -35 mV at pH 7, Cammack, 1982). The midpoint potential of the 3+/2+ transition of the Ni ion in inactive enzyme is -145 mV (pH 7.2) for the *D. gigas* enzyme (Cammack, 1982) and -115 mV (pH 8.0) for the *A. vinosum* enzyme (Coremans, 1992a, 1992b). The midpoint potentials of the Fe-S clusters in the *A. vinosum* enzyme, used in this study, are similar to those in the *D. gigas* enzyme (Coremans, 1992a, b). The *A. vinosum* hydrogenase contains an extra, unknown n=1 redox component (X, E_m' = +150 mV) (Coremans, 1992b). Thus, upon gradual oxidation of fully reduced enzyme, the two [4Fe-4S] clusters are oxidised first. Subsequently, provided the temperature is sufficiently high (Coremans, 1989, 1992b), the Ni ion in the active site is oxidised to the 3+ state and then the [3Fe-4S] cluster is oxidised. Finally, X is oxidised to a paramagnetic state, resulting in a strong coupling of X with the 3Fe cluster and a weak coupling of the [{[3Fe-4S]}\textsuperscript{1+}=X\textsuperscript{2+}] moiety with the Ni paramagnet (Surerus, 1994). The Fe ion in the active site is not redox active and remains in a low-spin, 2+ valence state (Surerus, 1994; Dole, 1996).

Two EPR signals from Ni are observed in oxidised, inactive [NiFe] hydrogenase of *A. vinosum*; the corresponding states are termed ‘unready’ (Ni\textsuperscript{'}\textsubscript{i}, g_\textsubscript{un} = 2.31, 2.24, 2.01) and ‘ready’ (Ni\textsuperscript{'}\textsubscript{i}, g_\textsubscript{un} = 2.33, 2.16, 2.01). Both signals are due to low-spin 3d\textsuperscript{3} systems of Ni\textsuperscript{'}\textsubscript{i} (Albracht, 1994). Reduced, active enzyme in the Ni\textsubscript{i}-C' state (subscript a for active) shows an EPR spectrum with g values 2.20, 2.15 and 2.01 (Van der Zwaan, 1985). When illuminated at low temperatures (T < 100 K) this state rapidly converts to the Ni\textsubscript{i}-L' state, with g_\textsubscript{un} = 2.045, 2.13, 2.27 / 2.29 (Van der Zwaan, 1985). The illumination effect is 6-fold slower when performed in D\textsubscript{2}O, indicating the photodissociation of a hydrogen species (Van der Zwaan, 1985). The formal valence state of the Ni ion in these two latter states has long been a topic of debate. For both states Ni\textsuperscript{'}\textsubscript{i} (3d\textsuperscript{3}) as well as Ni\textsuperscript{'}\textsubscript{i} (3d\textsuperscript{3}) ions have been proposed (Van der Zwaan, 1985; Moura, 1982; Kojima, 1983; Roberts, 1994). We presently believe that the Ni ion is trivalent in the Ni\textsubscript{i}-C' state and that upon illumination a metal-hydride bond in the active site is broken (Happe, 1999). The two electrons from this hydride are accepted by the Ni ion, reducing it to the 1+ state (the Ni\textsubscript{i}-L' state) and the remaining proton is accommodated somewhere in the vicinity of the active site. Carbon monoxide is a competitive inhibitor of most [NiFe] hydrogenases (K_i~25 μM). When exposed to CO the Ni\textsuperscript{'}\textsubscript{i} ion in the active enzyme binds CO (the Ni\textsubscript{i}-S-CO state) (Van der Zwaan, 1986). Low-temperature (20 K) FTIR spectra of [NiFe] hydrogenase from *A. vinosum* in the Ni-
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S-CO state show an extra absorption band at 2060 cm\(^{-1}\) ascribed to terminally bound, external CO (Bagley, 1994). The binding of CO to the active site has previously been used to study the magnetic properties of the Fe-S clusters and the unknown redox group X in the oxidised states, whilst keeping the Ni site EPR silent (Surerus, 1994).

In the present study, a novel form of the active site was encountered by the replacement of the CO gas by Ar in the presence of excess oxidising agent. From the properties of this form we conclude that the Ni-Fe site is apparently trapped in an active-like conformation in which the Ni ion experiences a ligand field very similar to that in the Ni\(_{4}\)-C\(^{-}\) state. Despite the spectral similarities with the Ni\(_{4}\)-C\(^{-}\) state, the novel form is inactive in a hydrogen uptake assay and hence named Ni\(_{4}\)-C\(^{-}\) (subscript i for inactive). The Ni\(_{4}\)-C\(^{-}\) state is also not light sensitive which suggests that it does not bind a photolabile hydrogen.

The formation of the new form has also been followed by FTIR. A quantitative analysis of the data showed that the amount of abstracted reducing equivalents during the oxidation of the enzyme widely exceeded the calculated amount. It is hypothesised that, under certain conditions, the [NiFe] hydrogenase from *A. vinosum* can oxidise CO to CO\(_{2}\).

**MATERIALS AND METHODS**

**Enzyme purification and characterisation.** *A. vinosum* (DSM 185) was grown in a 700-L batch culture (Van Heerikhuizen, 1981) in a medium essentially as described (Hendley, 1955; Albracht, 1983). Cells were harvested and the enzyme was isolated and purified as described (Coremans, 1989). Hydrogen oxidising activity was measured amperometrically (Coremans, 1992b). Protein concentrations were determined with the method according to (Bradford, 1976). Nickel concentrations were determined using Atomic Absorption Spectroscopy (AAS).

**Preparation of the novel enzyme form.** The purified [NiFe] hydrogenase from *A. vinosum* (200 μl, 20 μM in 50 mM Tris-HCl pH 8.0) was rebuffered to 50 mM MES (pH 6.0) and fully reduced by incubation under 100% H\(_{2}\) at 50°C for 45 min in a 10 ml serum-capped bottle. The sample was left to cool to RT for 10 min. Then the gas phase was exchanged with CO by evacuation and flushing (8 times) and the sample was left to equilibrate at RT for 10 min. The sample was then cooled on ice (10 min) and a K\(_{4}\)Fe(CN)\(_{6}\) solution (potassium ferricyanide, \(E_{m}^\prime = +358 \text{ mV}\)), also saturated with CO, was added anaerobically to a final concentration of 15 mM. The dye was left to react with the enzyme for 30 min, keeping the sample on ice. This was followed by exchanging CO with Ar (evacuation/flushing, 8 times), while still cooling the sample on ice for 30 min. At this point the protein solution had become slightly turbid, possibly due to the slightly acidic pH. To circumvent this, the same treatment was tried at higher pH values. At pH 8.0, however, this led to a considerable amount of the enzyme in the 'unready' state (Ni\(_{4}\)_4 to 50% of the EPR detectable centres), while oxidation at pH 9.0 mainly yielded enzyme in the oxidised 'ready' (Ni\(_{4}\)_2) state. To separate the redox dye from the enzyme, the sample was centrifuged aerobically through a Sephadex G 50 column at RT (Penefski, 1979). In this step the precipitate was also removed and the sample was rebuffered to 50
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mM Tris-HCl (pH 8.0). The clean sample was concentrated, transferred to an EPR tube and frozen under aerobic conditions. After inspection by EPR the sample was thawed, reduced with dithionite (25 mM) in the EPR tube under Ar and refrozen anaerobically. The whole process was also followed with FTIR. A solution of activated hydrogenase (~120 μM (11 mg/ml) in 50 mM Tris-HCl, pH 8.0) was exposed to CO gas in a vial as described above. Then an ice-cold CO-saturated ferricyanide solution was added (final concentrations: ferricyanide 7.5 mM, hydrogenase 5.6 mg/ml) and the cold mixture was quickly transferred to a gas-tight FTIR cell at RT. After the required flushing of the sample compartment of the spectrometer for 10 min with dry air, recording of the first FTIR spectrum was started exactly 15 min after the addition of the ferricyanide solution to the sample. From this point onwards, spectra were taken after 30, 45, 60, 75, 100, 120 and 150 min. Since the sample was saturated with CO at approximately 0°C, the calculated initial CO concentration in the measurement cell was 1.5 mM. The ferricyanide and ferrocyanide concentrations in the sample were determined from the peak areas (expressed in absorption units) using control spectra from stock solutions as standards (0.043 mM⁻¹ for ferricyanide and 0.312 mM⁻¹ for ferrocyanide in the used cell). In a control experiment bovine serum albumin (BSA, 5.5 mg/ml) and ferricyanide (7.5 mM) were incubated under CO and monitored for 150 min with FTIR.

Spectroscopic analysis. X-band EPR spectroscopy, illumination of the samples, simulation and integration of spectra were performed as before (Happe, 1999). FTIR spectra were taken on a BioRad FTS 60A spectrometer equipped with an MCT detector. Averages of 100 spectra were recorded with a resolution of 2 cm⁻¹ using the spectrum of a buffer solution as a background. The sample temperature was 25°C. Samples were loaded into a gas-tight transmission cell consisting of CaF₂ windows and Teflon spacers (path length 50 μm). The software supplied by BioRad was used to determine peak surface areas.
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RESULTS

EPR spectroscopy. After the treatment described in the experimental section, the active-site nickel was paramagnetic. Its EPR spectrum ($g_{\text{opt}} = 2.200, 2.160, 2.009$, Figure 1, trace a) was very similar to the well-studied spectrum of enzyme in the reduced, active, Ni$_x$-C state ($g_{\text{opt}} = 2.195, 2.145, 2.008$, Figure 1, trace b) (Van der Zwaan, 1985). Since the new form was inactive (see Activity measurements) it was named Ni$_x$-C$_{ox}$ (i for inactive, ox for oxidised). At 14 K the EPR spectrum of the new form also showed an intense absorption around $g = 2$ from an oxidised [3Fe-4S] cluster (Figure 1, trace c). Part of the cluster population showed the typical strong coupling to the unknown redox component X (see arrows) (Surerus, 1994), but contrary to customary oxidised enzyme (Albracht, 1984), there was no detectable coupling of the [3Fe-4S]=X$^+$ moiety to the Ni paramagnet. The $g = 2$ signal looked precisely like the one found in oxidised enzyme where the Ni ion was trapped in the EPR-silent state with CO (Surerus, 1994). The intensity of the Ni signal represented 0.35 spins/Ni and was approximately 70% of the intensity of the combined [3Fe-4S] cluster and the [3Fe-4S]=X$^+$ moiety.

![Figure 1. X-band EPR spectra of different forms of the [NiFe] hydrogenase of A. vinosum: (a) the novel form in the oxidised state; inset: enlargement (5x) of the low-field part of the spectrum showing a small amount of enzyme in the Ni$^+$ state ($g_x = 2.31, g_y = 2.24$), (b) the Ni$_x$-C state, (c) the oxidised [3Fe-4S] cluster of the sample in (a) recorded at 14 K; inset: enlargement (4x) recorded at a modulation amplitude of 0.4 mT, (d) the novel form, anaerobically reduced with 25 mM dithionite, (e) the Ni$_x$-L state, (f) the novel form, anaerobically reduced and incubated under CO recorded at 30 K, (g) reduced, active enzyme incubated under CO and recorded at 45 K. EPR conditions: microwave frequency, 9,427 MHz; temperature, 70 K (unless stated otherwise); power, 2 mW; modulation amplitude, 1.27 mT.](image-url)
Despite the similarities with the Ni\textsubscript{1-}C\textsuperscript{−} state, the Ni\textsubscript{1-}C\textsubscript{m}\textsuperscript{−} state was not sensitive to light at cryogenic temperatures; illumination with white light for more than 20 min at 4.2, 30 or 70 K did not invoke any change in the spectrum (not shown). For comparison, the Ni\textsubscript{1-}C\textsuperscript{−} state completely converted to the Ni\textsubscript{1-}L\textsuperscript{−} state within 5 min when illuminated at 30 K (g\textsubscript{em} = 2.041, 2.127, 2.266 / 2.290, Figure 1, trace e) (Van der Zwaan, 1985). Likewise, the Ni\textsubscript{1-}C\textsubscript{m}\textsuperscript{−} state was not sensitive to O\textsubscript{2} or CO: 30 min incubation at 50°C under air or CO did not alter the spectrum (not shown). Also a short exposure (20 s) to 1% H\textsubscript{2} / 99% He at 2°C did not re-establish the light-sensitivity, so if the new form can react with H\textsubscript{2}, its affinity for hydrogen can not be very high.

The novel form could be reduced chemically with dithionite (25 mM under Ar, 20 min at RT) to the Ni\textsubscript{1-}C\textsubscript{m}\textsuperscript{−} state (red for reduced). The EPR spectrum changed in a similar manner as the spectrum of the Ni\textsubscript{1-}C\textsuperscript{−} state upon illumination, but the resulting spectrum was more complex; a summation of four slightly different spectra was observed. While only one g\textsubscript{y} value was observed (2.045), four partly overlapping g\textsubscript{x} values and three clearly resolved g\textsubscript{z} values at 2.19, 2.24 and 2.30 were distinguished. The g\textsubscript{y} at 2.24 is asymmetric and probably consists of two overlapping peaks (Figure 1, trace d). The overall pattern, especially the g\textsubscript{y} value, was fairly similar to the Ni\textsubscript{1-}L\textsuperscript{−} spectrum (Figure 1, trace e).

After chemical reduction, the novel form was incubated under CO yielding the Ni\textsuperscript{1-} CO state with an almost axial spectrum (g\textsubscript{em} = 2.112, 2.096, 2.010, Figure 1, trace f). A very similar spectrum was observed after prolonged incubation of reduced, active enzyme under CO, but this signal represented only 2% of the total enzyme concentration (Figure 1, trace g) (Van der Zwaan, 1986). The intensities of the spectra of the Ni\textsubscript{1-}C\textsubscript{m}\textsuperscript{−} state (Figure 1, trace d) and the Ni\textsuperscript{1-} CO state (Figure 1, trace f) were the same, indicating a stoichiometric conversion of one into the other upon binding of CO. Only a very weak light-sensitivity was observed for the Ni\textsuperscript{1-} CO state: after 45 min of illumination with white light at 30 K some 10% ended up in the state before CO treatment. The remaining part still showed the axial spectrum.

**FTIR spectroscopy.** Due to instrumental limitations the first FTIR spectrum could only be taken 15 min after the start of the reaction with ferricyanide (Figure 2, trace a). It showed the [NiFe] hydrogenase from *A. vinosum* in mainly the CO-inhibited state. The stretch vibration from the intrinsic CO was at 1929 cm\textsuperscript{−1} and from its intensity an enzyme concentration of 61 μM (5.6 mg/ml) was estimated. The region where the three other peaks that belong to this state occur, is enlarged in Figure 2: two peaks were observed at 2068 and 2081 cm\textsuperscript{−1} from the active-site cyanides and a third one at 2055 cm\textsuperscript{−1} from the exogenous, inhibiting CO. The two intense bands came from ferricyanide (FIG, 2115 cm\textsuperscript{−1}) and ferrocyanide (FOC, 2037 cm\textsuperscript{−1}). Part of the added ferricyanide (initial concentration: 7.5 mM) was already reduced after 15 min. The intensity of the observed peak at 2037 cm\textsuperscript{−1} corresponded to a ferrocyanide concentration of 0.58 mM; the ferricyanide concentration after 15 min was 6.89 mM. The changes in the hydrogenase active site and those in ferricyanide and
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Figure 2. Oxidant-induced changes of the CO-inhibited [NiFe] hydrogenase of A. vinosum followed in time by FTIR spectroscopy. Spectra are not baseline corrected. FIC, potassium ferricyanide; FOC, potassium ferrocyanide.
Spectra taken after 15 min (a), 45 min (b) and 150 min (c).

Ferrocyanide concentrations could now be followed in time, monitoring the absorption peaks of all reactants involved.

From trace b in Figure 2 it can be seen that after 45 min at 25°C, the ferricyanide concentration further decreased to 6.73 mM and the ferrocyanide concentration rose to 0.72 mM. The peak at 1929 cm\(^{-1}\) from the Ni\(_5\)-S-CO state decreased and two overlapping peaks at 1948 cm\(^{-1}\) and 1945 cm\(^{-1}\) appeared. The peaks of the intrinsic enzyme-bound cyanides diminished in time. After 150 min the 1929 cm\(^{-1}\) peak had completely disappeared while the 1948 / 1945 cm\(^{-1}\) band as well as that of the ferrocyanide had further increased (Figure 2, trace c). After 150 min the ferrocyanide and ferrocyanide concentrations were 6.52 mM and 0.95 mM, respectively. Close inspection of the cyanide region showed two low-intensity, broad peaks at 2078 and 2091 cm\(^{-1}\). Analysis of the ferricyanide and the ferrocyanide concentrations in time (Figure 3) clearly showed that the ferri-compound was reduced to the ferro-compound. As the enzyme concentration was 61 µM (5.6 mg/ml), the steady increase of the ferrocyanide concentration to 950 µM showed that the source of the reducing equivalents could not only be the enzyme. In the control experiment with BSA (5.5 mg/ml) and 7.5 mM ferricyanide saturated with CO, no reduction of ferricyanide to ferrocyanide was observed which showed that the extra electrons were not produced by a non-specific background reaction.

The measured EPR samples (prepared at pH 6.0 and rebuffered to pH 8.0) were concentrated and inspected by FTIR as well. They showed an absorption peak of the intrinsic CO at 1948 cm\(^{-1}\) with a shoulder at 1945 cm\(^{-1}\) (not shown). No clear peaks were observed in the cyanide region. Upon reduction of this species with dithionite (25 mM) the CO peak shifted to 1938 cm\(^{-1}\) and decreased threefold in intensity (not shown). Again, no clear peaks were observed in the cyanide region.
Activity measurements. The novel form in its oxidised state showed no immediate activity in the uptake assay, despite the EPR-spectral resemblance to active enzyme. After a lag phase of about 1 to 2 min a slow increase in activity was observed to a maximum specific activity of only 1.8 U/mg (untreated, activated enzyme: 164 U/mg). This residual activity is probably due to a small amount of enzyme that was oxidised to one of the normal oxidised states (see Figure 1, trace a). After 2 h incubation under H₂ at RT the specific activity had not increased any further, indicating that the enzyme was irreversibly inactivated.

DISCUSSION

EPR spectroscopy. Since the EPR spectrum of the new form in the oxidised state (the Ni₅-S-CO state) and that of enzyme in the Ni₅-C state are much alike in terms of g values and line shapes (Figure 1), we conclude that the coordination of the Ni ion must be highly similar in both. The final step in the production of the new form was an oxidation of the divalent nickel in the Ni₅-S-CO state with ferricyanide, which could only occur upon removal of CO by flushing with Ar. Hence, the formal oxidation state of the Ni ion in the new state is most likely 3+, and the EPR signal suggests a low spin 3d⁷ system. This resembles the properties of the formal trivalent state of Ni in the Ni₅-C state (Van der Zwaan, 1986). Since the gₓ value is very close to the free electron g value (2.0023), the unpaired electron is in an orbital with mainly dₓ²-character (Wertz, 1972). Assuming octahedral symmetry this implies that the dₓ²⁻orbit is the one highest in energy.

Untreated *A. vinosum* enzyme often shows a coupling of part of the [3Fe-4S]⁺ cluster population with an unidentified redox component X (Coremans, 1992b; Albracht, 1984). The [3Fe-4S]⁺=X⁰ moiety is weakly coupled to the S = 1/2 system of Ni³⁺ (Surerus, 1994). Although coupling of X to the 3Fe-cluster is observed (as indicated by the arrows in trace c, Figure 1) there is no interaction with the paramagnetic Ni. This is obviously due to a change in the environment of Ni in the oxidised state of the new form compared to enzyme in the Ni₅⁺ and Ni₅⁻ states. Single-crystal EPR on the [NiFe]
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hydrogenase from \textit{Desulfovibrio vulgaris} Miyazaki F showed that in the oxidised states the \( g \) axis runs from Ni to one of the bridging thiols (Trofanchuk, 2000) (Cys533, numbering of the residues is as in the \textit{D. gigas} crystal structure. Volbeda. 1995). EPR studies on the \(^7\)Se-enriched F\(_{\infty}\)-non-reducing hydrogenase from \textit{Methanococcus voltae} in the Ni\(_{-}\)-C\(^*\) state suggested that in this state the \( g \) axis points to the terminal SeCys530 (Sorgenfrei, 1997). The \( g \)-tensor of the Ni\(^{1+}\) ion in the new form, with its EPR spectrum strongly resembling that of the Ni\(_{-}\)-C\(^*\) state, is most probably similarly oriented (i.e. the \( g \) axis pointing from Ni to Cys530).

Contrary to enzyme in the Ni\(_{-}\)-C\(^*\) state, the EPR signal of the Ni\(_{-}\)-C\(_{\text{red}}\) state did not change upon illumination with white light. Reduction with dithionite to the Ni\(_{-}\)-C\(_{\text{red}}\) state, however, yielded a spectrum resembling that of the Ni\(_{-}\)-L\(^*\) state. Since the change in spectrum was induced by a chemical reduction, the Ni ion in the reduced novel form is accordingly proposed to be formally 1+ (3d\(^9\)).

These observations support the Ni(III) – Ni(I) transition proposed in the model for the photolysis described earlier (Happe, 1999). Recent relativistic DFT calculations also support a formal Ni(III) – Ni(I) transition (Stein, 2001). Since the \( g \) value (2.045) of the Ni\(_{-}\)-L\(^*\) spectrum deviates considerably from \( g \), the unpaired electron is expected to be in an orbital with predominantly \( d_{z^2} \) character (Wertz, 1972). This means that the \( d_{z^2} \) orbital is the orbital highest in energy in both the dark and the light state. In the \(^7\)Se-enriched \textit{M. voltae} hydrogenase in the Ni\(_{-}\)-C\(^*\) state the superhyperfine coupling of the unpaired electron (3d\(^7\), unpaired electron in \( d_{z^2} \)) with the \(^7\)Se nucleus (\( I = 1/2 \)) is anisotropic and mainly interacts in the \( g \) direction (Sorgenfrei, 1997). Deducing from this, the orientation of the \( g \) axis is along the Ni-SeCys530 bond. In the Ni\(_{-}\)-L\(^*\) state, however, the \(^7\)Se nucleus couples mainly isotropically to the unpaired electron (3d\(^7\), unpaired electron in \( d_{z^2} \)). A finite electron density on the \(^7\)Se nucleus in the Ni\(_{-}\)-L\(^*\) state then suggests a 90° flip of the magnetic axes on going from the dark to the light state. The formation of a light-insensitive, oxidised Ni\(_{-}\)-C\(^*\) state raises the question whether the photolabile hydrogen species in the Ni\(_{-}\)-C\(^*\) state is a direct ligand to Ni or not. Current views in (especially) theoretical approaches (for a recent overview see Fan, 2001 and references therein) predict that the photolabile hydrogen species is in a bridging position between Ni and Fe. We feel that the removal of a hydride ion (an excellent nucleophile) from Ni would result in major changes in the EPR spectrum. It cannot be excluded, however, that the presence or absence of a bridging hydride has only minimal effects on the Ni EPR spectrum.

Nonetheless, the superhyperfine interaction observed in a model complex with an axially coordinated hydrogen atom (\( I = 1/2 \)) to a paramagnetic Ni(I) site was incomparably larger (~300 MHz) (Morton, 1984) than the magnetic interactions observed in the [NiFe] hydrogenases from \textit{A. vinosum} (Van der Zwaan, 1985) and \textit{Thiocapsa roseopersicina} in the Ni\(_{-}\)-C\(^*\) state (Whitehead, 1993). Both studies report an interaction between the Ni-based unpaired electron and the photolabile hydrogen species of only 20 MHz. This rules out an axial hydride positioned along the magnetic z-axis. In our view a likely binding place for the hydrogen species in the Ni\(_{-}\)-C\(^*\) state would be at the active-site Fe\(^{2+}\) ion, trans to the active-site CO, but without Fermi-contact interaction with Ni.
Figure 4. Schematic overview of the spectroscopic characteristics, the proposed structural models and the formal valencies of Ni in the novel states described in this report. Orientations of the $g$-axes are based on a combination of the data presented here and those deduced from the study on the Fe$_{ex}$-nonreducing [NiFeSe] hydrogenase from M. voltae enriched in $^{75}$Se (Sorgenfrei, 1997).

Finally in this section, we would like to discuss the binding of CO to the chemically reduced Ni$_1$-C$_{red}$ state to form the Ni$^+$-CO state. A nearly axial EPR spectrum with the same integrated intensity as the spectrum of the reduced state of the novel form was the result. The line shape was exactly the same as the EPR spectrum observed after prolonged incubation of active, reduced enzyme under CO (Van der Zwaan, 1985). These signals are also reminiscent of an EPR signal observed in carbon monoxide dehydrogenase (CODH) from *Clostridium thermoacetica* after incubation under CO, that was attributed to a Ni(III)-based unpaired electron interacting with CO (and with Fe in $^{57}$Fe-enriched enzyme) (Ragsdale, 1982). A recent crystal structure of a CODH from *Carboxydothermus hydrogenoformans* revealed a novel [Ni-4Fe-5S] cluster as the active site (Dobbek, 2001). In this cluster the Ni ion is in a tetrahedrally distorted square planar geometry, about 0.3 Å above the plane spanned by four of the inorganic S ligands (at ~2.3 Å). CO binding is suggested to occur at the apical position, imposing a square pyramidal geometry on the Ni ion (Dobbek, 2001). The X-ray structure from the CODH of *Rhodospirillum rubrum* was recently published as well and in this work the active site was proposed to consist of a [Ni-3Fe-4S] cluster bridged to a mononuclear Fe site by a cysteine sulphur (Drennan, 2001).

The nearly axial EPR spectrum of the Ni$^+$-CO state might also arise from a Ni ion in a square pyramidal geometry, binding four cysteine sulphurs and at least one end-on bound CO molecule (Figure 4c). The fact that only the reduced form of the novel form can bind CO, leads us to the conclusion that the resulting EPR spectrum is caused by an unpaired electron based at a monovalent Ni site. A paramagnetic, CO binding state (Ni$^+$-CO) displaying a rhombic spectrum ($g_{ex} = 2.12, 2.07, 2.02$) has been observed before in the [NiFe] hydrogenase from *A. vinosum* (Van der Zwaan, 1986). Rapid-mixing/rapid freezing studies indicated that also in this state the Ni ion is formally
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monovalent (Happe, 1999). The Ni$_i^{+}$CO state was formed through the illumination at 30 K of the Ni$_i$-C' state with CO very close to the active site. Illumination led to the formation of the regular Ni$_i$-L' state and after dark adaptation the rhombic spectrum of the Ni$_i^{+}$CO state was observed. The major difference between the Ni$_i^{+}$CO and the Ni$_i^{+}$CO state is that in the first state the active site remained intact (as can be seen from the light-sensitivity) whereas for the second state the insensitivity to light and the lack of H$_2$ oxidising activity indicated an irreversible change in the active site. We currently do not understand the nature of this change in the active site, but from the loss of intensity of the cyanide absorption bands in the FTIR it looks as if the iron side is seriously damaged. In both the Ni$_i^{+}$CO ($g_m = 2.070$) and the Ni$_i^{+}$CO state ($g_m = 2.073$) the shift of g values towards 2 indicates an increase in ligand field compared to the Ni ion in the Ni$_i$-L' state ($g_m = 2.152$). This can be explained by the coordination of a strong ligand like CO to the Ni$^{3+}$ ion. We cannot exclude the possibility of two CO molecules binding to Ni, stabilising an octahedral coordinated metal centre.

Higher quality FTIR spectra are required to further address this possibility.

**FTIR spectroscopy.** Figure 2 shows that the oxidation of the Ni$_i$-S-CO state could very well be followed with FTIR since all the reactants involved absorb in the 1800-2200 cm$^{-1}$ region. It can clearly be seen that the reduction of ferricyanide (Figure 3) is accompanied by an oxidation of the [NiFe] hydrogenase, supporting the idea that the final species (with an EPR spectrum very similar to that of the Ni$_i$-C' state) is in the 3+ state. Furthermore, the observed CO peak at 1948 cm$^{-1}$ and the shoulder at 1945 cm$^{-1}$ were also observed in the FTIR spectra of the concentrated samples used to measure EPR (not shown). The shoulder at 1945 cm$^{-1}$, together with the small peaks in the cyanide region at 2091 and 2078 cm$^{-1}$ probably reflect a portion of enzyme in the Ni$_i$' state. A fair amount (up to 40% of the EPR detectable spins) of Ni$_i$' was also observed in the EPR spectrum as a by-product of the anaerobic oxidation when performed at pH 8.0 (not shown). The vibrational frequencies (1945, 2078 and 2091 cm$^{-1}$) are in reasonable agreement with earlier published data for enzyme in the Ni$_i$' state (Happe, 1999).

The smaller absorptions in the cyanide region quickly declined during the anaerobic oxidation. Together with the lack of activity, the impossibility of reactivation and the occurring precipitates after anaerobic oxidation, this points to an irreversible process of inactivation, probably due to major changes at the Fe site in the active site. The Ni site, however, remains (at least partly) in an intact conformation, as can be concluded from the EPR spectra, although the amount of spins is clearly substoichiometric.
CO-oxidising activity of [NiFe] hydrogenases? Since the enzyme concentration in the FTIR experiment was 61 μM, the concentration of reducing equivalents that could maximally be supplied by the oxidation of the Ni$_{5}$-S-CO state is 305 μM. Nevertheless, a comparison of the FTIR spectra in time shows that almost 1.0 mM of ferricyanide is reduced to ferrocyanide, suggesting that yet another process is involved in the production of electrons (Figure 3).

One obvious source of electrons available in the reaction mixture is CO, that can be oxidised to CO$_2$ according to the reaction $\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{H}^+ + 2\text{e}^-$ which is commonly catalysed by CODH. The electrons produced in this reaction can be used to reduce ferricyanide to ferrocyanide. Since there are about 700 μM of reducing equivalents produced in excess of what the reduced enzyme could deliver, 350 μM of CO could be oxidised to CO$_2$. Our tentative conclusion is that the enzyme can possibly catalyse the oxidation of CO. The reverse has been shown before when the CODH from Clostridium thermoaceticum was reported to display a weak hydrogenase activity (Menon, 1996).

Contamination by a co-purified CODH is not very likely as these are generally strongly inhibited by low cyanide concentrations ($K_L \sim 1$ μM) (Menon, 1996). The high ferricyanide concentration (7.5 mM) will automatically yield a considerable background concentration of free cyanide, high enough to inhibit any contaminating CODH. The control experiment with BSA shows that the extra electrons are not produced by a non-specific background reaction. In spite of the rather high concentration of CO remaining after 150 min (calculated concentration: 1.15 mM), the ferricyanide reduction decreases in time. We think this is due to the oxidation of the enzyme to the Ni$_{5}$-C$_m$' state, an irreversibly inactivated state.

A comparison of the EPR - and the FTIR experiments. There was one notable difference between the production of the new form for the EPR measurements and the process of oxidation as studied with FTIR: the amount of CO gas molecules per enzyme molecule. In the preparation of the EPR sample the CO-inhibited enzyme was mixed with ferricyanide under 1 bar of CO. The volume of the gas phase was 10 ml (446 micromoles) of CO. The amount of enzyme in this incubation was 4 nanomoles and thus the enzyme / CO ratio was 1:112,000. The only possible way to oxidise the active site under these conditions was by removal of CO by exchanging the gas phase for Ar.

The enzyme / CO ratio in the FTIR experiment was quite different: in the measurement cell (12 μl) there was only CO in solution (1.5 mM or 18 nanomoles) and the amount of enzyme was 0.7 nanomoles (61 μM). Hence, the ratio in the FTIR experiment was 1:25. Comparing this to the ratio in the EPR experiment illustrates the difference in CO pressure. This explains why oxidation of the sample was easier under the conditions maintained in the FTIR experiment.

A few general remarks about the observed redox states. The authors want to stress that the valencies as proposed for the active-site Ni must be considered formal valencies. Extensive XAS studies on the A. vinosum [NiFe] hydrogenase have shown that the energy of the Ni K-edge in the different redox states does not change accordingly (Davidson, 2000). Actually, with just one exception, the observed K-edges are within 1 eV the same in all redox states. This implies that the overall electron density at the Ni-site does not change much. In model systems one-electron redox changes result in shifts of K-edge energies of typically ~2 eV (Kirby, 1981). The succession of EPR-detectable and EPR-silent
states indicates several one-electron processes in the reduction of the active site. This implies that in the more oxidised states (the formal Ni\textsuperscript{2+} states) quite some electron density is donated from the ligands to the Ni ion, whereas in the lower oxidation states (formally Ni\textsuperscript{2+} and Ni\textsuperscript{3+}) the electron density flows in the opposite direction from the ion to the ligands. The net result of this process is that the one electron changes occurring on the whole of the active site result in a fairly constant electron density on the Ni and subsequent small shifts in K-edge energies. This might actually very well be the function of the Fe(CN)\textsubscript{3}(CO)-site: a buffer for electron density, allowing fast H\textsubscript{2} turnover at the Ni site.

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