Infrared Characterization of the Bidirectional Oxygen-Sensitive [NiFe]-Hydrogenase from E. Coli

Moritz Senger¹, Konstantin Laun¹, Basem Soboh², and Sven T. Stripp¹,*

¹ Department of Physics, Experimental Molecular Biophysics, Freie Universität Berlin, 14195 Berlin, Germany; sven.stripp@fu-berlin.de
² Department of Physics, Genetic Biophysics, Freie Universität Berlin, 14195 Berlin, Germany; basem.soboh@fu-berlin.de
* Correspondence: sven.stripp@fu-berlin.de; Tel.: +49-030-838-55069

Abstract: [NiFe]-hydrogenases are gas-processing metalloenzymes that catalyze the conversion of dihydrogen (H₂) to protons and electrons in a broad range of microorganisms. Within the framework of green chemistry, the molecular proceedings of biological hydrogen turnover inspired the design of novel catalytic compounds for H₂ generation. The bidirectional “O₂-sensitive” [NiFe]-hydrogenase from Escherichia coli HYD-2 has recently been crystallized; however, a systematic infrared characterization in the presence of natural reactants is not available yet. In this study, we analyze HYD-2 from E. coli by in situ ATR FTIR spectroscopy under quantitative gas control. We provide an experimental assignment of all catalytically relevant redox intermediates alongside the O₂- and CO-inhibited cofactor species. Furthermore, the reactivity and mutual competition between H₂, O₂, and CO was probed in real time, which lays the foundation for a comparison with other enzymes, e.g., “O₂-tolerant” [NiFe]-hydrogenases. Surprisingly, only Ni-B was observed in the presence of O₂ with no indications for the “unready” Ni-A state. The presented work proves the capabilities of in situ ATR FTIR spectroscopy as an efficient and powerful technique for the analysis of biological macromolecules and enzymatic small molecule catalysis.

Keywords: Metalloenzymes; Spectroscopy; Small Molecules

1. Introduction

Hydrogenase are gas-processing metalloenzymes that catalyze “hydrogen turnover” (H₂ → 2 H⁺ + 2 e⁻) in all kingdoms of life [1]. Oxidation of molecular hydrogen is typically referred to as “H₂ uptake” while proton reduction leads to “H₂ release”. Under physiological conditions [FeFe]-hydrogenases show pronounced H₂ release activity while [Fe]- and [NiFe]-hydrogenases are typically employed in H₂ uptake [2–4]. These three classes are unrelated and differ significantly in protein fold and composition of the catalytic transition metal cofactor [5]. An understanding of the molecular proceedings of hydrogen turnover may inspire novel catalytic compounds for an industrial generation of H₂ as a fuel [6–8].

The gram-negative, facultative anaerobic bacterium Escherichia coli synthesizes at least three membrane-associated [NiFe]-hydrogenases [9]. Facing the cytoplasm, HYD-3 catalyzes the oxidation of formic acid into H₂ and CO₂ as part of the formate hydrogenlyase complex. HYD-1 and HYD-2 are respiratory hydrogenases of the periplasm whose hydrogen turnover activity has been suggested to be linked to the energy metabolism of the cell (HYD-2) or the reduction of trace amounts of O₂ (HYD-1) [10]. Accordingly, HYD-1 is an “O₂-tolerant” [NiFe]-hydrogenase that maintains significant turnover activity in the presence of O₂ while HYD-2 is inhibited by O₂ and has been classified “O₂-sensitive” [11–13]. In O₂-sensitive [NiFe]-hydrogenases the structural placidity of an iron-sulfur cluster proximal to the active site cofactor was shown to play a key role in the immediate recovery from O₂-inhibited species and reduction of O₂ under hydrogen turnover conditions [14–16].

Figure 1a depicts the recently crystallized HYD-2 heterodimer [13]. Subunit HybO (~40 kDa) carries three iron-sulfur clusters that facilitate electron exchange between redox partners and catalytic
center. In difference to HYD-1 [17,18] the moiety most proximal to the [NiFe] cofactor is a conventional [4Fe-4S]-cluster. Subunit HybC (~63 kDa) binds the catalytic center in tunneling distance to the proximal iron-sulfur cluster (Fig. 1b). Four conserved cysteine residues (C61/C64, C546/C549) coordinate the bimetallic [NiFe] active site. The iron ion shows two cyanide (CN−) and one carbon monoxide ligand (CO), which facilitates infrared (IR) spectroscopic studies on [NiFe]-hydrogenases [19]. The CO/CN− stretching frequencies are sensitive “reporters” for changes in electron density distribution across the cofactor and can be addressed to characterize different redox- and protonation states [20–22]. In the second coordination sphere, several conserved residues have been proposed to be involved in proton transfer between bulk water and active site cofactor. One possible trajectory includes C546 and E14 [23–25], an alternative route comprises R479, D103, and D544 [26].

Protein crystallography, electrochemistry, EPR- and IR spectroscopy on [NiFe]-hydrogenases significantly contributed to the understanding of biological hydrogen turnover [27–30]. The H2 uptake reaction was suggested to include 3 – 4 redox species (Fig. 2) [20,21]. Ni-SI represents the active-ready, oxidized state (Ni2+/Fe3+) that stabilizes an open coordination site between nickel and iron ion (dotted circle). Dihydrogen reacts at the nickel ion and forms a Ni-Fe bridging hydride species in the “super-reduced” Ni-R state [31,32]. One proton is released in the process; for Ni-R1, high resolution crystallography suggested a protonated cysteine (C546 in HYD-2) [25] but protonation of R479 has also been proposed [26]. The very location of the proton in Ni-R2 and Ni-R3 is unclear; however, the shift to lower IR frequencies from R1 to R3 may indicate an increase in distance relative to the Fe(CN):CO reporter group.

Oxidization and deprotonation of Ni-R by one electron forms the Ni-C state [33]. The assignment of Ni2+/Fe3+ in Ni-R and Ni3+/Fe2+ in Ni-C is reflected by a CO frequency up-shift of up to ~40 cm−1 for Ni-R3, compensated by the presence of a proton in Ni-R1 (~Δ15 cm−1) and Ni-R2 (~Δ30 cm−1) [20]. Both Ni-R and Ni-C carry a bridging hydride. The release of this hydride is accompanied by a two-fold reduction of the cofactor in the Ni-L states (Ni4+/Fe3+) and protonation of the protein.

Figure 1. Crystal structure of the [NiFe]-hydrogenase HYD-2 from E. coli (pdb coordinates 6EHQ) [13]. (A) Subunit HybC (light grey) binds the catalytic cofactor whereas subunit HybO (red) carries three iron-sulfur clusters. In this representation HybO is truncated by 48 amino acids (*) to reveal the view onto the active site niche (black circle). (B) Catalytic cofactor including amino acids of the first and second coordination sphere (cyan) that are potentially involved in proton transfer [23–26]. Non-bonded molecules that may be water (crosses) are potentially involved in proton transfer, too.
fold [34,35]. Infrared data on Ni-L2 and Ni-L3 is rare; however, existing studies suggest a down-shift relative to Ni-C by ~Δ70 cm⁻¹ and ~Δ85 cm⁻¹, respectively [36–39]. This is compatible with a formal difference of two electrons. Oxidation and deprotonation restores Ni-SI and completes the cycle as shown in Fig. 2. Whether Ni-L and/or Ni-C are included is subject to ongoing discussions [20,21].

Figure 2. Intermediates of the [NiFe] cofactor and catalytic cycle [20,21]. Based on crystallographic data the cartoon shows the bimetallic cofactor as coordinated by four cysteines. The iron ion is ligated by two CN⁻ (blue) and one CO ligand (red). Catalytic intermediates Ni-SI, Ni-R, and Ni-C/Ni-L constitute for the H₂ uptake reaction. In the active-ready oxidized state Ni-SI the cofactor reacts with CO and O₂ to form the inhibited species Ni-SCO and Ni-A/B, respectively.

Figure 2 additionally depicts on the reaction of [NiFe]-hydrogenases with CO and O₂. Carbon monoxide binds the terminal coordination site of Ni²⁺ in the Ni-SI state to form the oxidized, CO-inhibited Ni-SCO state [40]. This species differs from the parent Ni-SI state by a weakly coordinated Ni-CO ligand at high IR frequencies [41]. In the presence of O₂, two different O₂-inhibited, “super-oxidized” states may be enriched: Ni-A and Ni-B (Ni³⁺/Fe²⁺). Ni-A represents an “unready” species that converts slowly into Ni-SI while Ni-B is readily activated under reducing conditions [42,43]. [NiFe]-hydrogenases that do not form Ni-A maintain catalytic activity under aerobic conditions and have been classified O₂-tolerant [14]. Standard [NiFe]-hydrogenases, e.g., as isolated from strict anaerobes are inhibited by O₂ and have been referred to as O₂-sensitive [11]. Both Ni-A and Ni-B carry a Ni-Fe bridging ligand (most likely a hydroxo species) that reacts to H₂O upon reductive activation [44–46]. On structural level the kinetic activation differences between Ni-A and Ni-B remain elusive.

In this work we present the first conclusive IR characterization of the periplasmatic [NiFe]-hydrogenase HYD-2 from E. coli. In difference to HYD-1 [37], only tentative IR band assignments have been reported for HYD-2 [47,48]. We suggest an experimentally verified assignment of catalytic (Ni-SI, Ni-C, Ni-R) and inhibited states (Ni-SCO, Ni-B) making use of steady-state and real-time attenuated total reflection Fourier-transform infrared spectroscopy (ATR FTIR) under gas control. The reaction and mutual competition with H₂, CO, and O₂ is probed in kinetic experiments and forms the basis for a comparative analysis with other [NiFe]-hydrogenases. Our methodology has mainly been applied to [FeFe]-hydrogenases in the past [49–51]. Here, we show that all advantages (low sample demand, simple experimental design, measurements under biological conditions, etc.) hold true for the analysis of [NiFe]-hydrogenases as well.
2. Results and Discussion

2.1 FTIR steady-state and in situ difference spectra

Synthesized and isolated in the absence of O₂, HYD-2 from E. coli adopted a steady-state mixture of at least two species (Fig. 3a, first spectrum “as-isolated”). The carbonyl stretching frequencies are distinct enough (νCO = 1966 and 1945 cm⁻¹) whereas the cyanide regime from 2100 – 2040 cm⁻¹ does not immediately suggest four individual νCN⁻ contributions. In the presence of 1% CO ambient partial pressure, the as-isolated spectrum completely converted into the oxidized, CO-inhibited state Ni-SCO (Fig. 3a, second spectrum) [41]. This species is characterized by a high-frequency Ni-CO band (νCO = 2054 cm⁻¹) and a low-frequency Fe-CO band (νCO = 1944 cm⁻¹). The CN⁻ stretching frequencies can be found at 2084 and 2073 cm⁻¹. Isotope editing with ¹³CO confirmed the existence of two individual, vibrationally uncoupled CO ligands at the active site cofactor (Fig. S1). When as-isolated HYD-2 was brought in contact with 1% O₂ (Fig. 3a, third spectrum), an oxygen-inhibited state was populated (νCO = 1957 cm⁻¹ with νCN⁻ = 2092 and 2082 cm⁻¹) that has been suggested to be Ni-B by Hexter and co-workers [47]. Incubation under 100% O₂ for up to four hours did not induce any further changes in the spectrum. In the presence of 1% H₂ ambient partial pressure the species observed in the as-isolated sample were found to be significantly diminished (Fig. 3a, fourth spectrum). Even under 100% H₂ these states did not vanish completely (Fig. S2). Three novel CO bands were detected at 1950, 1936, and 1927 cm⁻¹ alongside a larger number of cyanide bands.

In order to refine the band assignments, we recorded in situ difference spectra as a function of gas. For this an as-isolated absorption spectrum was subtracted from the absorption spectrum after the respective gas treatment (e.g., “H₂ – as-is”). The first spectrum in Fig. 3b indicates an enrichment of the 1945 cm⁻¹ band over the 1966 cm⁻¹ band upon elongated treatment with N₂ gas (very slow, the spectrum depicts a treatment with N₂ of more than four hours). This allows correlating CO and CN⁻ frequencies as suggested in the Fig. 3b and Tab. 1. The enrichment under auto-oxidizing conditions (an inert gas is exploited to remove traces of evolved H₂ that otherwise would back-react with the enzyme [50]) indicates that the 1945 cm⁻¹ band represents the active-ready, oxidized state Ni-SI. This assignment is supported by the spectral similarity to Ni-SCO (Fig. 3a) [41]. The concomitant decrease of the 1966 cm⁻¹ band under oxidizing conditions suggests a reduced cofactor intermediate.

![Figure 3](image-url)
According to the analyses of Ash et al. [20], the difference in CO stretching frequency of 21 cm\(^{-1}\) to Ni-SI strongly supports an assignment to the one-electron reduced hydride state Ni-C. Interestingly, the second spectrum in Fig. 3b shows the loss of Ni-SI and Ni-C in the presence of CO although it is known that only Ni-SI is sensitive to CO inhibition [41]. Most likely, this reflects changes in the steady-state equilibrium between Ni-C and Ni-SI upon CO reacting with the later: once CO binds to the oxidized cofactor, reduced enzyme converts into Ni-SI and becomes reactive to CO. This process is related to intramolecular electron transfer and auto-oxidation of Ni-C into Ni-SI as discussed above, however much more effective because the CO affinity outrivals the H\(_2\) release activity of HYD-2 [52], in particular under non-reducing conditions.

Under O\(_2\), HYD-2 is oxidized into either Ni-A or Ni-B (Fig. 3b, third spectrum). The difference spectrum shows a pronounced loss of Ni-C and Ni-SI, as well as a species at lower frequencies (\(v_{CO} = 1911\) cm\(^{-1}\) with \(v_{CN} = 2062\) and 2052 cm\(^{-1}\)) that may represent an Ni-L state [34,35]. As HYD-2 is an O\(_2\)-sensitive [NiFe]-hydrogenases, it is not immediately possible to conclude whether Ni-A or Ni-B was formed. In the presence of H\(_2\), the difference spectrum represents the enrichment of at least three novel species (Fig. 3b, fourth spectrum). The corresponding three CO bands are clearly visible in the difference spectrum, and the downshift relative to Ni-C suggests an assignment of Ni-R1 (1950 cm\(^{-1}\)), Ni-R2 (1936 cm\(^{-1}\)), and Ni-R3 (1927 cm\(^{-1}\)) [32]. In Fig. S2, the correlation of CO and CN\(^{-}\) bands for the R-states is presented.

2.2 Kinetic traces for the reaction with H\(_2\), CO, and O\(_2\):

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Repeated Ni\(_2\)/H\(_2\) cycles on HYD-2 from *E. coli*. The sum of peak area of CO/CN\(^{-}\) bands assigned to each state is plotted against time. In the presence of 1% H\(_2\) ambient partial pressure, Ni-C and Ni-SI are diminished in favor of the “super-reduced” R-states. The one-electron reduced hydride state Ni-C (dark grey traces) is the dominating species throughout the experiment. The experiment demonstrates the robust nature of the Ni-C/Ni-SI ↔ Ni-R1 conversion.

Figure 4 illustrates the reaction of HYD-2 to changes of the N\(_2\)/H\(_2\) gas composition in the head phase above the protein film. The sum of peak area of one CO and two CN\(^{-}\) bands for each species is followed over time. In as-isolated sample, Ni-C and Ni-SI were populated in a ratio of approximately 2:1. When the inert N\(_2\) atmosphere was enriched with 1% H\(_2\) ambient partial pressure a steep decrease of both these species was observed; however, while Ni-SI vanished from the spectrum completely Ni-C lost only ~40% sum of peak area and remained to be the most prominent species. Ni-R1 rose in the presence of H\(_2\) over Ni-SI and Ni-C as dominant “super-reduced” state while Ni-R2 and Ni-R3 are omitted for clarity (see Fig. S2 for the complete data set). Removal of H\(_2\) resulted in a slow conversion of the R-states back into Ni-C and Ni-SI, compared to the fast decrease under H\(_2\). Ni-SI only reaches ~50% sum of peak area in comparison to as-isolated sample. No traces of Ni-A, Ni-B, or Ni-SCO were observed.
Figure 5. Inhibition of HYD-2 from *E. coli* with CO in either N₂ or H₂ carrier gas. (a) With 1% CO in N₂ carrier gas HYD-2 converts into Ni-SCO immediately (red trace). The species was semi-stable in the absence of CO (100% N₂) adopting an equilibrium with Ni-SI (blue trace). Under 1% H₂ an immediate conversion into the catalytic species Ni-C and Ni-R was observed. (b) In contrast, using H₂ as carrier gas it takes up to ~30% CO to convert half of the enzyme population into Ni-SCO (Fig. S3).

Figure 5 depicts the inhibition and reactivation kinetics upon contact with 1% CO ambient partial pressure in either N₂ or H₂ carrier gas. The left panel shows how Ni-C and Ni-SI were populated under N₂ and converted into Ni-SCO at 1% CO. After 500 s, CO was removed from the gas phase, which prompted the film to swing into an equilibrium between Ni-SCO and Ni-SI. The CO-inhibited species remained to be the dominant, though. After 500 s, 1% H₂ was introduced to the gas stream and gave rise to Ni-C/Ni-R over Ni-SCO/Ni-SI. The reaction of CO with the reduced active site cofactor is addressed in the right panel of Fig. 5. Here, the percentage of CO was stepped up systematically in H₂ carrier gas. Half-max intensity for Ni-SCO is achieved at ~30% CO (Fig. S3) whereas only 1% CO resulted into a full conversion under N₂ (left panel). The observed decline of reduced states cannot be explained by the relative decrease of H₂ ambient partial pressure between 100 – 50%: Fig. 4 and Fig. S2 showed that 1% H₂ is sufficient for a reduction of HYD-2, therefore no significant changes in the effective H₂ redox potential can be assumed. Similar to the equilibrium between Ni-C and Ni-SI (see above) the R-states are in equilibrium with the “unprotected” Ni-SI state under reducing conditions (Fig. 5, right panel). In the presence of CO Ni-SI reacts to Ni-SCO, which induces a continuous auto-oxidation of the reduced species into Ni-SI (note the relative stable population of Ni-SI up to 10% CO). The enrichment of Ni-C and Ni-R under H₂ affects this equilibrium and delays the process of auto-oxidation. This results in an indirect protection against CO inhibition although H₂ and CO do not compete for the same binding site (compare Fig. 2) [40]. No reaction with CO is observed in the absence of Ni-SI, i.e. under O₂ (Fig. S3).
Figure 6. Inhibition of HYD-2 from E. coli with O₂ in either N₂ or H₂ carrier gas. (a) With 1% CO in N₂ carrier gas HYD-2 converts into Ni-B immediately (green trace). Ni-B is stable under 100% N₂ and converted in the presence of 1% H₂ into reduced species Ni-C/Ni-R within minutes. (b) The mutual robustness of the hydride-binding species Ni-C and Ni-R shows in the right panel as well: Ni-B is enriched in the film only at 99 – 100% O₂. Potentially explosive mixtures of 15 – 85% O₂ in H₂ were avoided.

In a similar set of experiments, Fig. 6 depicts on the inhibition and reactivation kinetics of HYD-2 upon contact with 1% O₂ ambient partial pressure in the presence of either N₂ or H₂ carrier gas. Under O₂, Ni-C and Ni-SI were converted into a single species, i.e., Ni-A or Ni-B (left panel). In difference to the reaction with CO, no changes in equilibrium were observed upon removal of O₂ from the gas stream and the oxygen-inhibited species remained stable even in the absence of O₂. After 500 s, 1% H₂ was introduced to the gas stream, resulting in a conversion of the oxygen-inhibited species into the typical mixture of Ni-C and Ni-R as seen before (Fig. 4). Based on the apparent reactivity of the oxygen-inhibited species we propose to assign the identified IR signature under O₂ to Ni-B, in agreement with earlier suggestions [47]. HYD-2 was stable under 100% O₂ for at least four hours with no notable decrease or conversion into any other species, e.g., Ni-A.

In the next step, HYD-2 was subjected to increasing concentration of O₂ in the presence of H₂ carrier gas (Fig. 6, right panel). The system converted quantitatively into Ni-B not until 99 – 100% O₂ was reached. In difference to the reaction with CO, the lack of O₂ inhibition under reducing conditions can be explained by competition of O₂ and H₂ for the same binding site at the [NiFe] cofactor [40,53]. Interestingly, Ni-C is populated over Ni-R from 90 – 98% O₂ most likely reflecting the instability of the “super-reduced” R-states under increasingly oxidizing conditions. This hints at an equilibrium between hydrogenase- and oxygenase-activity that has been suggested to explain the hydrogen turnover activity of “O₂-tolerant” [NiFe]-hydrogenases [54]. Accordingly, the diverging reaction kinetics from CO- and O₂-inhibition as highlighted in Fig. 7 may indicate the difference between CO release (fast) and O₂ reduction (slow).
3. Materials and Methods

3.1 Synthesis and isolation of HYD-2 from E. coli

To synthesize and isolate preparative amounts of active [NiFe]-hydrogenase, 5 mL “reaction mix” were used. As reported earlier [48], this mixture contained $^{35}$S$^{35}$HybC (HYD-2 catalytic subunit) and the purified maturases HybG-HypDE (GDE complex) and HypEF. An “activation mix” comprising ATP, carbamoylphosphate, NiCl$_2$, FeSO$_4$, and sodium dithionite was added. After incubation at room temperature in the absence of air, cofactor synthesis was initiated adding endopeptidase HybD. After 30 minutes, the mixture was supplemented with HYD-2 subunit $^{16}$S$^{16}$HybO. Active $^{35}$S$^{35}$HybC-$^{16}$S$^{16}$HybO heterocomplex (HYD-2 holoenzyme) was isolated from the reaction mix by strep-tactin affinity chromatography and histidine affinity chromatography. The sample was concentrated to ~200 µM protein concentration in 100 mM Tris/HCl buffer (pH 8) including 5 mM dodecyl maltoside as detergent. See ref. [48] for further details.

3.2 Infrared spectroscopy

All spectroscopy was performed at room temperature and in the dark on a Bruker Tensor27 FTIR spectrometer housed in a CoyLab anaerobic chamber. A DuraSampIR 2 optical cell with a three-reflections silicon microrcrystal was used for ATR spectroscopy. Beam path, spectrometer, and anaerobic chamber were purged with dry N$_2$ gas as provided by an Inmatec nitrogen generator (gas purity 5.0). For each experiment, 1 µL of HYD-2 protein sample (~200 µM) was pipetted onto the silicon crystal, dried under N$_2$, and rehydrated in the presence of an aerosol by running the gas mixture through a wash bottle with a buffer solution of 10 mM Tris/HCl (pH 8). Digital mass flow controllers (Sierra) were used to adjust the absolute amount of gas and ratio between reactants. All gas treatments were performed at ambient partial pressure (1.013 bar). Following this procedure, concentrated and stable protein films were formed (Fig. A2). See ref. [50] for details of the experimental setup.
Data were recorded with a spectral resolution of 2 cm\(^{-1}\) (80 kHz scanner velocity) and 1.000 interferometer scans (steady-state spectra) or 25 scans when following kinetic traces. Difference spectra were calculated from single channel spectra via OPUS software. In order to analyze kinetic traces in the cofactor regime from 2150 – 1850 cm\(^{-1}\), absorption spectra were corrected for the background contribution of liquid water by a low frequency spline function using a home-written routine. The CO/CN\(^{-}\) signature of all redox species was trained on pure spectra to evaluate the individual frequencies, peak areas, and peak ratios. The “sum of peak area” as obtained by Gaussian fits was plotted to follow the conversion of species over time. In Fig. A1, this procedure is demonstrated on a representative data set.

4. Conclusions

The membrane-associated, bidirectional [NiFe]-hydrogenase HYD-2 from \textit{E. coli} has been analyzed by \textit{in situ} ATR FTIR spectroscopy. Based on the reactivity with \textit{H}_2, CO, and O\(_2\), an experimental band assignment is suggested that agrees well with other [NiFe]-hydrogenases (Tab. 1) [20]. In difference to HYD-1 from \textit{E. coli} [37], the one-electron reduced Ni-L states were barely observed and the formally isoelectronic hydride state Ni-C represents the most stable redox species. This suggests superior stabilization of the Ni-Fe bridging hydride in HYD-2 and may be related to the diverging catalytic properties of HYD-1 (H\(_2\) uptake) and HYD-2 (bidirectional) [52]. Furthermore, we have no reason to conclude that anything else than Ni-B was enriched in the presence of O\(_2\). Ni-A and Ni-B are difficult to distinguish by IR spectroscopy. However, in comparison to the O\(_2\)-sensitive hydrogenases of strict anaerobes that recover from O\(_2\) inhibition over the time course of hours [42], the spectral assignment to Ni-B is easily compatible with the fast reactivation kinetics of HYD-2 observed in our experiments. The crystal structure of HYD-2 clearly indicates a standard [4Fe-4S] cluster proximal to the [NiFe] cofactor [13], well in agreement with protein film electrochemistry on HYD-2 that prompted a classification as O\(_2\)-sensitive [52]. Thus, HYD-2 was expected to show low rates of reductive reactivation and form Ni-A upon reacting with O\(_2\). This is not the case. With respect to O\(_2\) tolerance, the role of the proximal iron-sulfur cluster in the membrane-bound [NiFe] hydrogenase of \textit{Ralstonia eutropha} has been questioned recently [55]. The example of HYD-2 from \textit{E. coli} shows that the reaction with O\(_2\) may involve additional check screws like proton- and electron transfer that remain to be evaluated.

<table>
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\(^1\) In the presence of \(^{13}\)CO the Ni-CO band shifts to 209 cm\(^{-1}\).

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Figure S1: \(^{13}\)CO isotope editing, Figure S2: Further H\(_2\) titrations and Ni-R band assignment, Figure S3: Further CO titrations.

**Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “conceptualization, S.T.S. and M.S.; methodology, S.T.S. and M.S.; formal analysis, S.T.S.; investigation, M.S. and K.L.; resources, B.S.; writing—original draft preparation, S.T.S.; writing—review and editing, M.S.; supervision, S.T.S.”

**Funding:** Basem Soboh acknowledges funding by the DFG priority program “FeS for life” (SPP-1927).
Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

![Figure A](attachment:figure_a.png)

**Figure A1.** Baseline correction, data fitting, kinetic description, and quality assurance at the example of the formation of Ni-B in the presence of O₂. All spectra are plotted from “as-isolated” (top) to “O₂-inhibited” (bottom). (a) Absorbance spectra (raw data, black) were corrected for the broad contribution of liquid water by spline functions (magenta). The CO- and CN– regime is indicated. (b) The Fe(CN)₂CO signature of the cofactor is described by three Gaussian fits including frequency, peak area (amplitude x width), and peak ratio. This data was obtained from pure spectra for Ni-SI (blue), Ni-C (dark grey), and Ni-B (green) beforehand. (c) The “sum of peak area” (CO + CN + CN) for each species is then plotted over the number of spectra. Depending on scanner velocity (80 kHz), spectral resolution (2 cm⁻¹), and number of averages (e.g. 25 interferometer scans), this value is converted into time. (d) Overlay of baseline-subtracted data (black) and fitted trace (envelope, red). (e) The residuals (“data – fit”) do not suggest any additional species and allow estimating the signal-to-noise ratio. (f) A difference spectrum between the last and first spectrum indicates a small increase of water (OH stretching, ~3500 cm⁻¹) that is accompanied by a decrease of protein signals (amide I, ~1655 cm⁻¹ and amide II, ~1540 cm⁻¹). These unspecific changes do not significantly affect the cofactor regime from 2150 – 1850 cm⁻¹ (marked).
Figure A2. Preparation of the protein film. (a) 1 µL protein solution of ~200 µM HYD-2 was pipetted to the silicon crystal and carefully “de-hydrated” under dry N₂. The spectra in the graph run from black (protein solution) to red (protein film). (b) Humidification of carrier N₂ induces a “re-hydration” of the protein film. This is an important prerequisite to follow the specific reactivity of HYD-2 varying gases. The spectra in the graph run from black (dry protein film) to blue (re-hydrated film). (c) Due to the overlap of water and protein bands, the changes in the film are not trivial to analyze. In particular, following the amide I band (~1655 cm⁻¹, open circles) and HOH bending mode intensity (~1635 cm⁻¹, open squares) is not helpful. The amide II band (1540 cm⁻¹, full circles) shares less overlap with liquid water and can be addressed to follow the changes in protein concentration more reliably. Upon de-hydration the band intensity increases by ~60 x 10⁻³ absorbance units while the decrease upon re-hydration accounts to only ~20 x 10⁻³ absorbance units. For a comparable hydration level, we observe a ~40 x 10⁻³ net-increase of amide II band intensity. (d) In the cofactor regime, the CO/CN⁻ become visible in the re-hydrated film (blue spectrum) where no such bands are observed with protein solution (black spectrum). Both spectra are baseline-corrected (see Fig. A1). The inferior signal-to-noise level in comparison to Fig. 3a stems from the number of averages (here: 50 interferometer scans).

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