Supporting Information

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

Moritz Senger 1, Konstantin Laun 1, Basem Soboh 2 and Sven T. Stripp 1,*

1 Department of Physics, Experimental Molecular Biophysics, Freie Universität Berlin, 14195 Berlin, Germany; senger@zedat.fu-berlin.de (M.S.); konstantin.laun@fu-berlin.de (K.L.)
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

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Figure S1. $^{13}$CO isotope editing. The black difference spectrum shows the enrichment of Ni-SCO over Ni-C and Ni-SI upon $^{12}$CO binding the nickel ion (see main script). In the presence of $^{13}$CO, the Ni-CO signal shifts from 2054 to 2009 cm$^{-1}$ (orange difference spectrum). The observed shift of 45 cm$^{-1}$ to lower frequencies is explained by the difference in reduced mass $\mu = \frac{m_1m_2}{m_1+m_2}$ between $^{12}$CO and $^{13}$CO. The Fe(CN)$_2$:CO signature remains unaffected by the isotope editing, which proves the presence of an individual CO ligand at the nickel site.
**Figure S2.** Further H₂ titrations and Ni-R band assignment. (a) In order to assign the Fe(CN):CO signature of the Ni-R states the following procedure was attempted. First, the sample was brought in contact with 1% O₂ to homogenize the film and enrich Ni-B over the as-isolated mixture of Ni-C and Ni-SI (see Figure 6 in the main script). Second, O₂ was replaced by 1% H₂ to reductively activate the enzyme. The reduced species Ni-C and Ni-R were found to dominate the spectrum with no increase of the oxidized Ni-SI in the presence of H₂. However, the H₂ – O₂ difference spectrum still included the negative bands of Ni-B. Although the film was completely reactivated with 1% H₂, a further increase of Ni-R over Ni-C was observed by switching the gas stream to 100% H₂. (b) “100% H₂ – 1% H₂” difference spectrum that depicts the increase of Ni-R1 – R3 over Ni-C. The Fe(CN):CO signature of Ni-C was identified before (see main script). Based on the singular peaks at 2076, 2054, and 2048 cm⁻¹ and the observation that both CO and CN⁻ bands shift in the same direction, a unique fit was achieved for the Fe(CN):CO assignment of Ni-R1 – R3 (see Table 1 in the main script). (c) This data was used to describe the concomitant increase and decrease of R-states, e.g., depending on the presence or absence of H₂ from the carrier gas.
Figure S3. Further CO titrations. (a) In the presence of O2 carrier gas, no CO inhibition was observed even when the gas stream was switched to 100% CO. The reaction with O2 very efficiently removes Ni-SI from the equilibrium of species. Therefore, CO cannot bind to the [NiFe] active site. (b) With N2 as carrier gas, 1% CO in the gas stream completely converts the film into Ni-SCO. The mixture of Ni-C and Ni-SI (characteristic for HYD-2 under inert gas) facilitates an immediate conversion into Ni-SCO in the presence of CO. (c) With H2 as carrier gas, an apparent decrease in the CO affinity is observed (see main script). (d) When the relative population of Ni-SCO is plotted against CO ambient partial pressure, the differences in CO affinity as a function of carrier gas can be visualized in a single graph.