
Stepwise assembly of the active site of [NiFe]-hydrogenase

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Supplementary Tables

Supplementary Table 1: Strains, plasmids, and oligonucleotides used in this study.

Strain/plasmid	Relevant characteristics	Source or reference
<i>Cupriavidus necator</i>		
H16	wild-type, MBH ⁺ , RH ⁺ , SH ⁺	DSM428
HF497	$\Delta hoxG$, $\Delta hypD$, $\Delta hoxH$; MBH ⁻ , RH ⁻ , SH ⁻	1
HF535	$\Delta hoxM$, $\Delta hoxH$; MBH ⁻ , SH ⁻	2
HF1065	$\Delta hoxK$, $\Delta hoxG$, $\Delta hoxC$, $\Delta hoxN$, $\Delta hoxH$; MBH ⁻ , RH ⁻ , SH ⁻	This work
HF1067	$\Delta hoxK$, $\Delta hoxG$, $\Delta hoxC$, $\Delta hoxN$, $\Delta hoxH$, $\Delta hypA2$; MBH ⁻ , RH ⁻ , SH ⁻	This work
HF1068	$\Delta hoxK$, $\Delta hoxG$, $\Delta hypA$, $\Delta hoxC$, $\Delta hoxN$, $\Delta hoxH$, $\Delta hypA2$; MBH ⁻ , RH ⁻ , SH ⁻	This work
HF1071	$\Delta hoxK$, $\Delta hoxG$, $\Delta hoxC$, $\Delta hoxH$; MBH ⁻ , RH ⁻ , SH ⁻	This work
<i>Escherichia coli</i>		
S17-1	Tra ⁺ recA, pro thi, hsdR chr:RP4-2	3
NEB [®] 5-alpha	Derivative of <i>E. coli</i> DH5 α ; cloning strain	New England Biolabs
Plasmids		
LITMUS 28	Ap ^r , lacZ', ColE1 ori	New England Biolabs
pEDY309	Tc ^r , RK2 ori, Mob ⁺	4
pCH420	0.75 kb PvuII fragment carrying $\Delta hypA1$ in pLO1	5
pCH579	3.1 kb BsaAI/SmaI fragment carrying $\Delta hypA2$ in PmeI site of pLO2	6
pCH659	1.5 kb Sall fragment carrying $\Delta hoxN$ in Sall site of pLO2	4
pGC15	P _{MBH} -hoxG in LITMUS 28 ^a	This work
pGC17	P _{MBH} -prohoxG in LITMUS 28 ^a	This work
pGC27	P _{MBH} -prohoxG in pEDY309 ^a	This work
pGC50	P _{MBH} -hoxG in pEDY309 ^a	This work
pLO1/pLO2	Km ^r , sacB, RP4 oriT, ColE1 ori	7
Oligonucleotides^b		
GC2	ATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAATC AGCTTACGCAACCCAAGGCTTC	
GC3	GGTGGCTCCAGCTAGCCATAACCTGTCTCCTAATTT CTGTATTGGTATTGGC	
P1	CACCTCGCGCCGTTTCGACG	
P2	GTGCCGGTATCGACGAGGCC	

^a All *hoxG* constructs contain a Strep-tag II-encoding sequence attached to the 5' end of the gene. ^b Oligonucleotides are depicted in the 5'-3' orientation and were purchased from Sigma-Aldrich.

Supplementary Table 2: $\nu(\text{CO})$ and $\nu(\text{CN})$ stretching frequencies related to the HoxG active site at different maturation stages and in different redox states.

Protein ^a	Redox state	$\nu(\text{CO})/\text{cm}^{-1}$	$\nu(\text{CN})/\text{cm}^{-1}$	Reference
(apo) preHoxG ^{ΔFeNi}	-	1929	2057, 2071 2071	This work
preHoxG ^{ΔNi}	-	1947 1962 1997	2072 2092	This work
procHoxG ^{ΔNi}	-	1947 1975 1997	2068 2077 2092	This work
preHoxG	-	1953	2066 2076 2090	This work
preHoxG ^S	-	1953	2066 2076 2090	This work, ⁸
mHoxG ^b	Ni _r -S _{II} Ni _r -S _I Ni _r -B'	1929 1939 1953 1958	2057, 2070 2057, 2070 2090	This work
procHoxG	Ni _r -S _{II} Ni _r -S _I	1929 1939 1950	2057, 2070 2057, 2070	This work
mHoxG _{ox} ^b	Ni _r -B'/'	1958 (broad)	2073, 2084	This work
mHoxG _{red} ^b	Ni _a -L	1889	2037, n.d.	This work
HoxC _{ai}	Ni _r -S _I Ni _r -S _{II}	1952 1941	2066, 2084 2064, 2077	⁹
HoxC _{ox}	Ni _r -B' Ni _r -B''	1969 1978	2081, 2090	⁹
HoxC _{red}	Ni _a -L	1889	n.d.	⁹

^a All HoxG (and HoxC) variants contain an N-terminal Strep-tag II for affinity purification. The only exception is preHoxG^S, whose C-terminal extension is equipped with a Strep-tag II at the very C terminus.⁸

^b Different redox states are exclusively observed for the mHoxG intermediate and compared with those of the recently characterized large subunit HoxC of the regulatory [NiFe]-hydrogenase (RH) from *C. necator*.

Abbreviations: ox = oxidized, ai = as-isolated, n.d. = not detected

Supplementary Table 3: Parameters obtained from the simulations of the Mössbauer spectra of the HoxG active site at different maturation stages and in different redox states.

Protein	Isomer shift (δ , mm s ⁻¹)	Quadrupole splitting (ΔE_Q , mm s ⁻¹)	Relative area (% total iron)	Ref.
preHoxG ^{ΔNi}	0.08 -0.08	1.02 1.05	78 22	This work
preHoxG	0.06 0.24	1.09 0.41	97 3	This work
mHoxG	0.14 0.05 1.49	0.62 1.10 3.52	71 22 7	This work
mHoxG _{ox}	0.17 0.06 1.49	0.61 1.12 3.52	71 21 8	This work
HoxC	0.14 1.43	0.65 3.02	92 8	⁹
HoxC _{ox}	0.15 1.43	0.59 3.13	90 10	⁹

The values of major species are shown in bold.

Supplementary Table 4: Comparison of preHoxG Fe K-edge EXAFS scattering models, fit simultaneously with the Ni K-edge EXAFS 3xNi-S1, 1xNi-Fe, 3xNi-C, 1xNi-S3 model. Debye-Waller factors (σ^2) were restrained to a range of 1.5-10.0 x 10⁻³ Å². Debye-Waller factors of Fe-C1 and Fe-C2 were reduced to a single parameter. The Ni-Fe/Fe-Ni distance and Debye-Waller factor was simultaneously fit to both Fe and Ni K-edge EXAFS as a set of common parameters. Errors are provided in parentheses. The best model is marked in bold.

Fe K-edge EXAFS						Ni K-edge EXAFS						
Model	Scatterer	N	R (Å)	σ^2 (10 ⁻³)	E_0 (eV)	Scatterer	N	R (Å)	σ^2 (10 ⁻³)	E_0 (eV)	Red. χ^2	R-factor
min.	Fe-C	3	1.93 (0.03)	4.9 (3.1)	7124 (3)	Ni-S1	3	2.22 (0.01)	4.4 (0.7)	8341 (2)	64	0.052
	Fe-S	2	2.34 (0.02)	3.9 (1.7)		Ni-Fe	1	2.78 (0.05)	10.0 (1.0)			
	Fe-Ni	1	2.78 (0.05)	10.0 (1.0)		Ni-C	3	2.93 (0.09)	1.5 (0.7)			
	Fe-C-N/O	6	3.08 (0.06)	7.5 (3.9)		Ni-S3	1	2.84 (0.08)	1.6 (0.5)			
w/Fe-C2	Fe-C1	2	1.93 (0.05)	2.5 (0.8)	7123 (3)	Ni-S1	3	2.22 (0.01)	4.4 (0.6)	8341 (2)	56	0.028
	Fe-C2	1	1.79 (0.06)			Ni-Fe	1	2.77 (0.05)	10.0 (1.0)			
	Fe-S	2	2.34 (0.02)	3.8 (1.8)		Ni-C	3	2.93 (0.08)	1.5 (0.7)			
	Fe-Ni	1	2.77 (0.05)	10.0 (1.0)		Ni-S3	1	2.84 (0.07)	1.6 (0.5)			
	Fe-C-N/O	6	3.08 (0.05)	7.5 (3.9)								
w/1xFe-S	Fe-C1	2	1.93 (0.05)	2.7 (1.1)	7123 (4)	Ni-S1	3	2.22 (0.01)	4.4 (0.7)	8341 (2)	78	0.60
	Fe-C2	1	1.79 (0.12)			Ni-Fe	1	2.77 (0.11)	10.0 (1.0)			
	Fe-S	1	2.34 (0.04)	1.5 (3.0)		Ni-C	3	2.93 (0.10)	1.5 (0.8)			
	Fe-Ni	1	2.77 (0.11)	10.0 (1.0)		Ni-S3	1	2.84 (0.09)	1.7 (0.6)			
	Fe-C-N/O	6	3.08 (0.06)	7.2 (4.7)								
w/Fe-O	Fe-C1	2	1.93 (0.19)	1.5 (4.0)	7123 (8)	Ni-S1	3	2.22 (0.01)	4.4 (0.7)	8341 (3)	85	0.036
	Fe-C2	1	1.80 (0.16)			Ni-Fe	1	2.77 (0.22)	10.0 (1.0)			
	Fe-O	1	2.03 (0.46)	4.2 (4.0)		Ni-C	3	2.93 (0.26)	1.5 (0.8)			
	Fe-S	2	2.34 (0.05)	3.7 (3.0)		Ni-S3	1	2.84 (0.29)	1.8 (0.6)			
	Fe-Ni	1	2.77 (0.22)	10.0 (1.0)								
	Fe-C-N/O	6	3.08 (0.16)	7.5 (4.7)								

Supplementary Table 5: Comparison of mHoxG Fe K-edge EXAFS scattering models, fit simultaneously with the Ni K-edge EXAFS 3xNi-S1, 1xNi-Fe, 3xNi-C, 1xNi-S3 model. Debye-Waller factors (σ^2) were restrained to a range of 1.5-10.0 x 10⁻³ Å². Debye Waller factors of Fe-C1 and Fe-C2 were reduced to a single parameter. The Ni-Fe/Fe-Ni distance and Debye-Waller factor was simultaneously fit to both Fe and Ni K-edge EXAFS as a set of common parameters. Errors are provided in parentheses. Best model is marked in bold.

Model	Scatterer	N	Fe K-edge EXAFS			Scatterer	N	Ni K-edge EXAFS			Red. χ^2	R-factor
			R (Å)	σ^2 (10 ⁻³ Å ²)	E_0 (eV)			R (Å)	σ^2 (10 ⁻³ Å ²)	E_0 (eV)		
min.	Fe-C	3	1.89 (0.02)	10.0 (5.0)	7122 (2)	Ni-S1	2	2.24 (0.01)	1.6 (0.3)	8341 (1)	28	0.021
	Fe-S	2	2.34 (0.01)	2.5 (0.7)		Ni-S2	1	2.41 (0.01)				
	Fe-Ni	1	2.83 (0.02)	8.3 (3.8)		Ni-Fe	1	2.83 (0.02)	8.3 (3.8)			
	Fe-C-N/O	6	3.04 (0.02)	8.3 (2.4)		Ni-C	3	3.13 (0.04)	9.9 (0.5)			
						Ni-S3	1	2.88 (0.07)	10.0 (5.0)			
w/Ni-O	Fe-C	3	1.89 (0.02)	10.0 (1.0)	7124 (2)	Ni-O	1	1.79 (0.02)	10.0 (5.0)	8336 (1)	30	0.017
	Fe-S	2	2.34 (0.01)	2.2 (0.6)		Ni-S1	2	2.22 (0.01)	1.5 (0.3)			
	Fe-Ni	1	2.76 (0.03)	10.0 (5.0)		Ni-S2	1	2.38 (0.01)				
	Fe-C-N/O	6	3.09 (0.03)	10.0 (5.0)		Ni-Fe	1	2.76 (0.03)	7.3 (1.3)			
						Ni-C	3	3.16 (0.04)	10.0 (5.0)			
w/Fe-O	Fe-C	3	1.91 (0.05)	9.5 (4.2)	7125 (2)	Ni-S3	1	2.71 (0.06)	10.0 (5.0)	8341 (1)	23	0.010
	Fe-O	1	2.10 (0.12)	2.7 (1.6)		Ni-S1	2	2.24 (0.01)	1.6 (0.3)			
	Fe-S	2	2.36 (0.04)	2.6 (1.0)		Ni-S2	1	2.41 (0.01)				
	Fe-Ni	1	2.83 (0.11)	8.1 (3.2)		Ni-Fe	1	2.83 (0.01)	8.1 (3.2)			
	Fe-C-N/O	6	3.06 (0.06)	10.0 (5.0)		Ni-C	3	3.13 (0.04)	10.0 (5.0)			
w/Fe-O & Ni-O	Fe-C	3	1.90 (0.05)	9.8 (4.6)	7125 (3)	Ni-S3	1	2.87 (0.06)	10.0 (5.0)	8336 (1)	28	0.010
	Fe-O	1	2.09 (0.11)	6.4 (4.6)		Ni-O	1	1.80 (0.02)	10.0 (5.0)			
	Fe-S	2	2.35 (0.01)	2.4 (1.0)		Ni-S1	2	2.22 (0.01)	1.5 (0.5)			
	Fe-Ni	1	2.77 (0.03)	7.0 (1.2)		Ni-S2	1	2.38 (0.01)				
	Fe-C-N/O	6	3.09 (0.04)	10.0 (5.0)		Ni-Fe	1	2.77 (0.03)	7.0 (1.2)			
						Ni-C	3	3.15 (0.05)	10.0 (5.0)			
						Ni-S3	1	2.73 (0.07)	10.0 (5.0)			

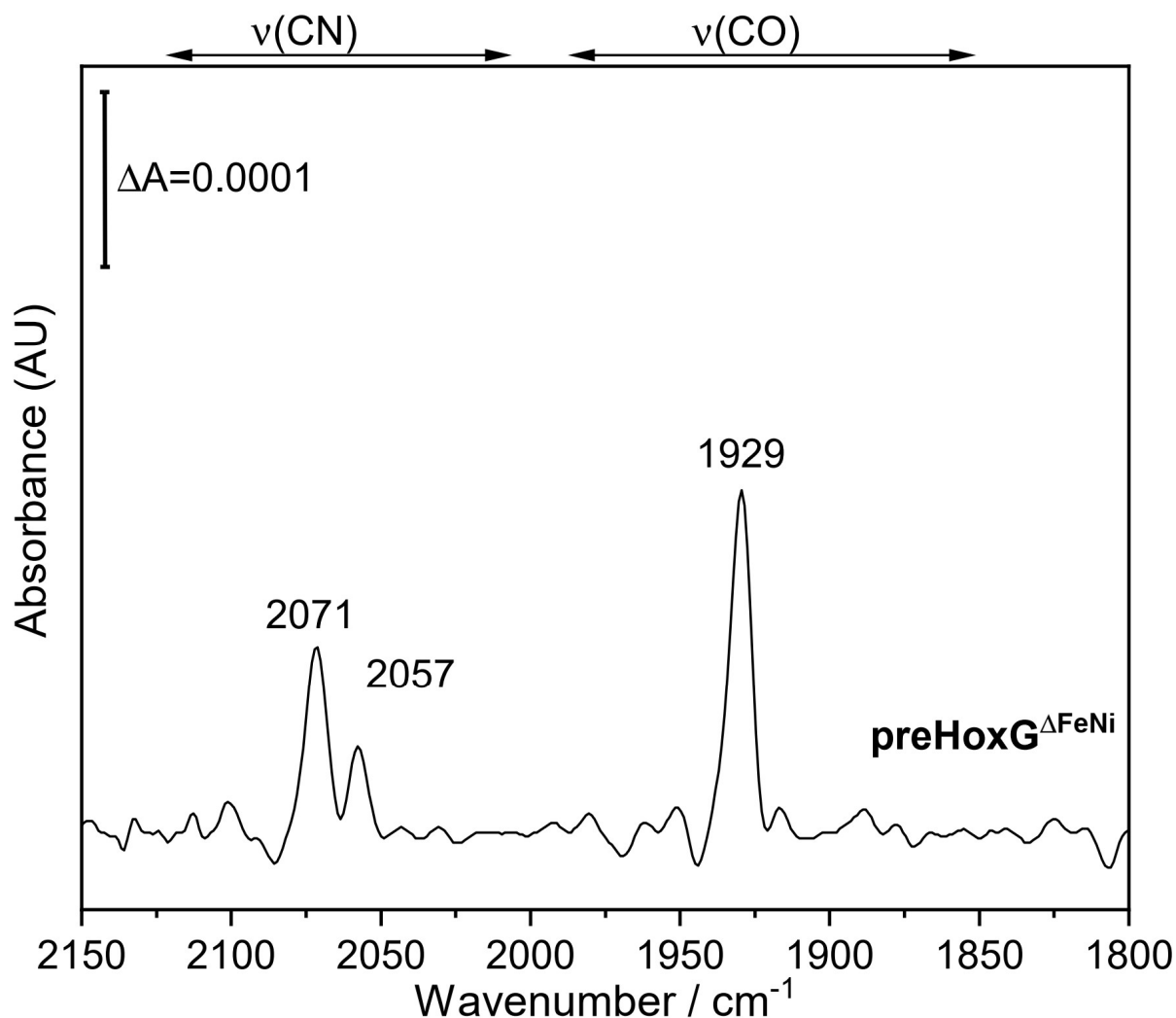
Supplementary Table 5: continued

Fe K-edge EXAFS						Ni K-edge EXAFS						
Model	Scatterer	N	R (Å)	σ^2 (10^{-3} Å 2)	E_0 (eV)	Scatterer	N	R (Å)	σ^2 (10^{-3} Å 2)	E_0 (eV)	Red. χ^2	R-factor
0.5 Ni-O	Fe-C	3	1.91 (0.03)	10.0 (5.0)	7125 (2)	Ni-O	0.5	1.79 (0.03)	9.9 (4.6)	8339 (1)	15	0.007
	Fe-O	1	2.11 (0.04)	3.4 (2.2)		Ni-S1	2	2.23 (0.01)	1.5 (0.2)			
	Fe-S	2	2.36 (0.01)	2.6 (0.7)		Ni-S2	1	2.40 (0.01)	7.5 (2.0)			
	Fe-Ni	1	2.82 (0.02)	7.5 (2.0)		Ni-Fe	1	2.82 (0.02)	9.9 (5.0)			
	Fe-C-N/O	6	3.07 (0.02)	10.0 (5.0)		Ni-C	3	3.12 (0.03)	10.0 (5.0)			
						Ni-S3	1	2.83 (0.06)	4.0 (1.0)			
no Ni-S2	Fe-C	3	1.90 (0.03)	10.0 (5.0)	7125 (2)	Ni-O	1	1.77 (0.01)	10.0 (5.0)	8330 (1)	14	0.008
	Fe-O	1	2.10 (0.05)	6.0 (0.6)		Ni-S1	2	2.21 (0.01)	3.5 (0.2)			
	Fe-S	2	2.35 (0.01)	2.4 (0.7)		Ni-Fe	1	2.78 (0.03)	1.7 (1.2)			
	Fe-Ni	1	2.78 (0.03)	10.0 (5.0)		Ni-C	3	3.12 (0.01)	4.0 (1.0)			
	Fe-C-N/O	6	3.09 (0.03)	10.0 (5.0)		Ni-S3	1	2.74 (0.02)				

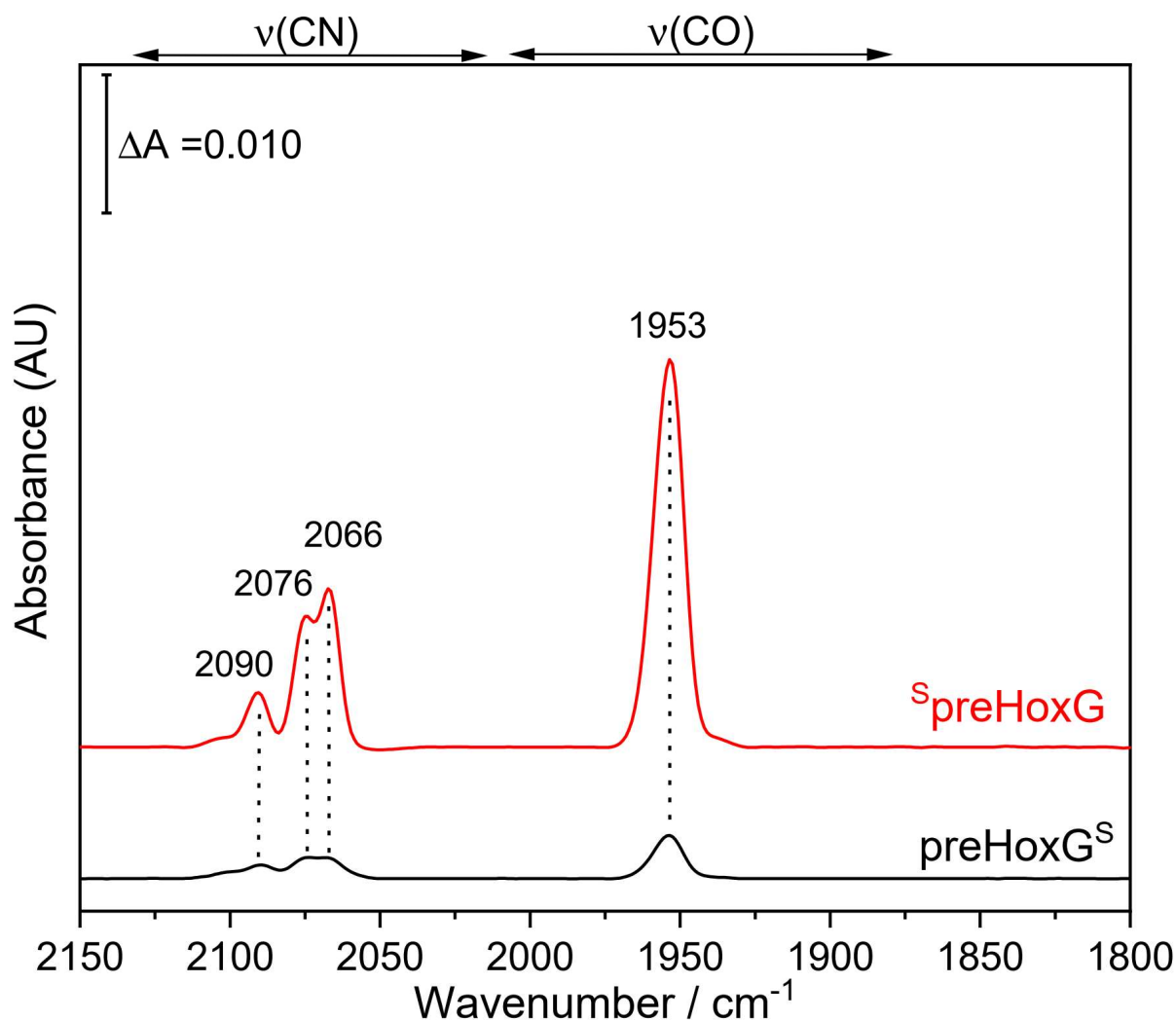
Supplementary Table 6. EPR-derived g values for mHoxG in different redox states.

Protein	Redox state	g1	g2	g3	Ref.
mHoxG	Ni _I -B' (<5%)	2.186	2.120	2.005	This work
mHoxG _{ox}	Ni _I -B'	2.186	2.120	2.005	This work
	Ni _I -B''	2.264	2.144	2.028	
		2.239	2.090	2.053	
mHoxG _{red}	Ni _a -L	2.336	2.121	2.057	This work
HoxC _{ox}	Ni _I -B'	2.203	2.132	2.004	⁹
	Ni _I -B''	2.246	2.164	2.025	
HoxC _{red}	Ni _a -L	2.305	2.095	2.053	⁹

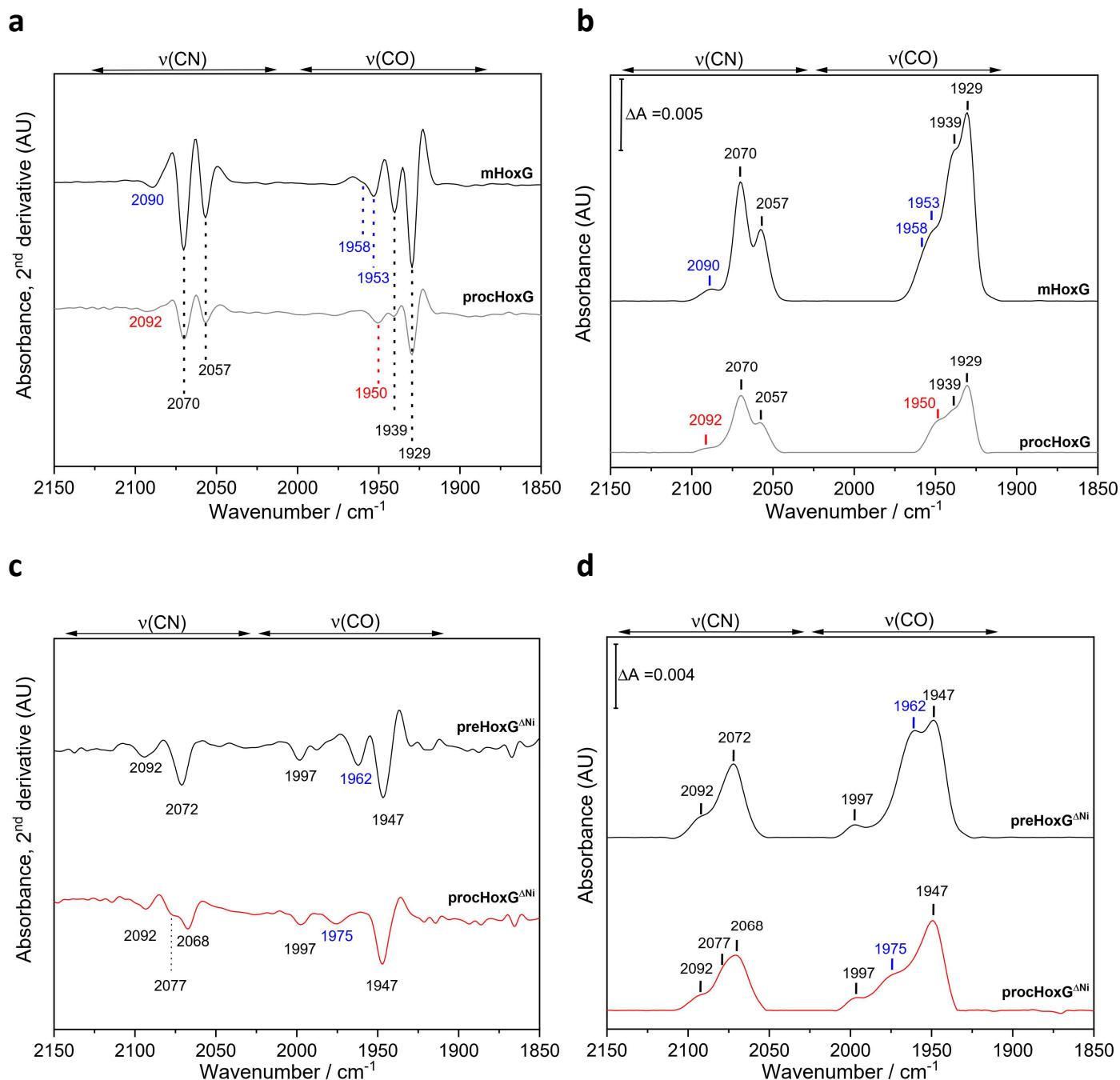
Supplementary Figures



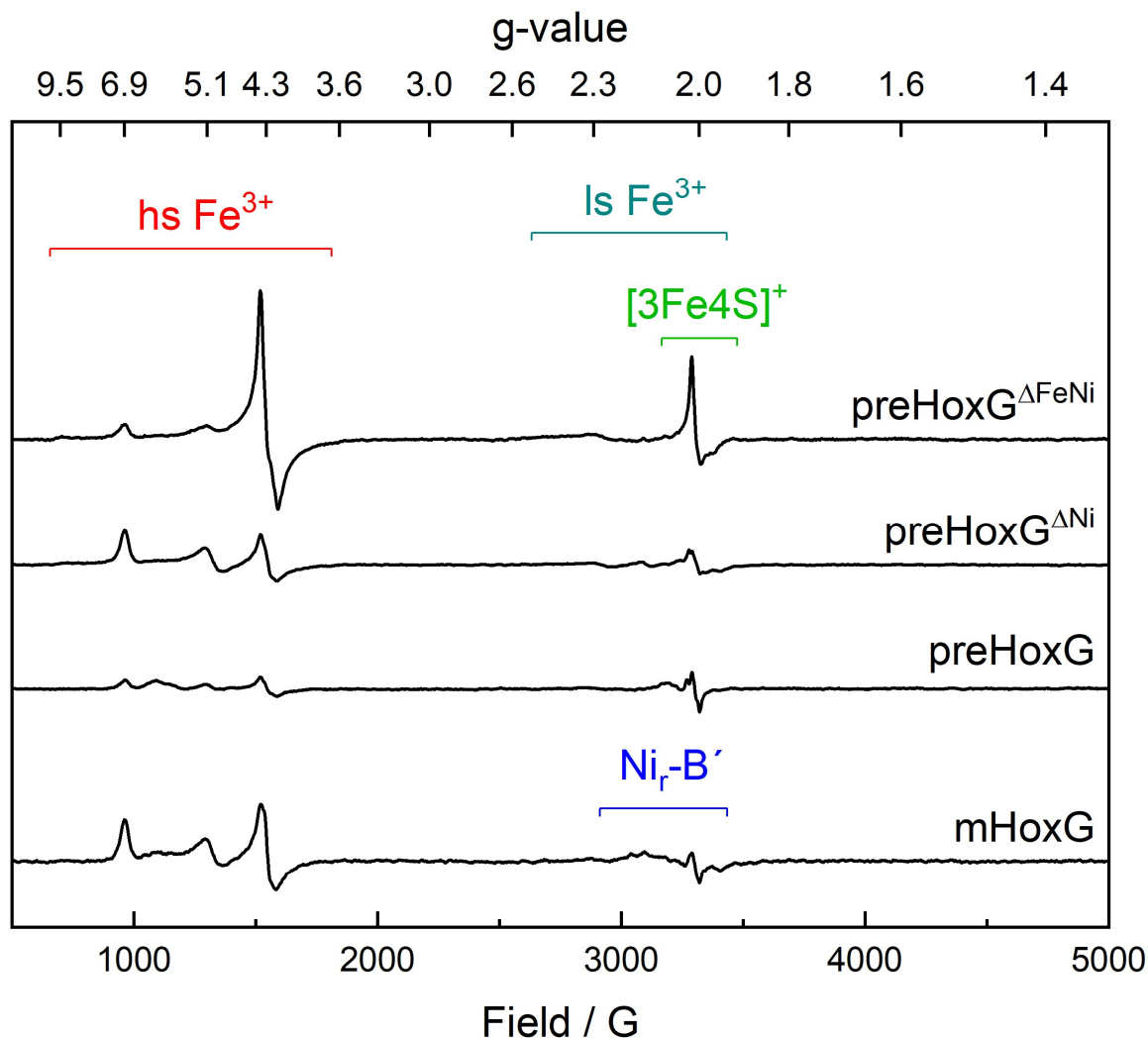
Supplementary Fig. 1: Infrared spectrum of *preHoxG*^{ΔFeNi}. The spectrum is an enlargement of the data shown in **Fig. 2c** to visualize the trace signals attributed to CO and CN stretching modes of miniscule $\text{NiFe}(\text{CN})_2(\text{CO})$ and/or $\text{Fe}(\text{CN})_2(\text{CO})$ active site populations loaded in *preHoxG*^{ΔFeNi} without the involvement of the maturase HypD. Normalizing the spectra of *preHoxG*^{ΔFeNi}, *preHoxG*, and *mHoxG* to the amide II bands, the intensities of the CO/CN stretching modes in *preHoxG*^{ΔFeNi} reflect less than 0.8 % cofactor incorporation. The IR experiments were performed with three independent samples and yielded consistent results (n=3).



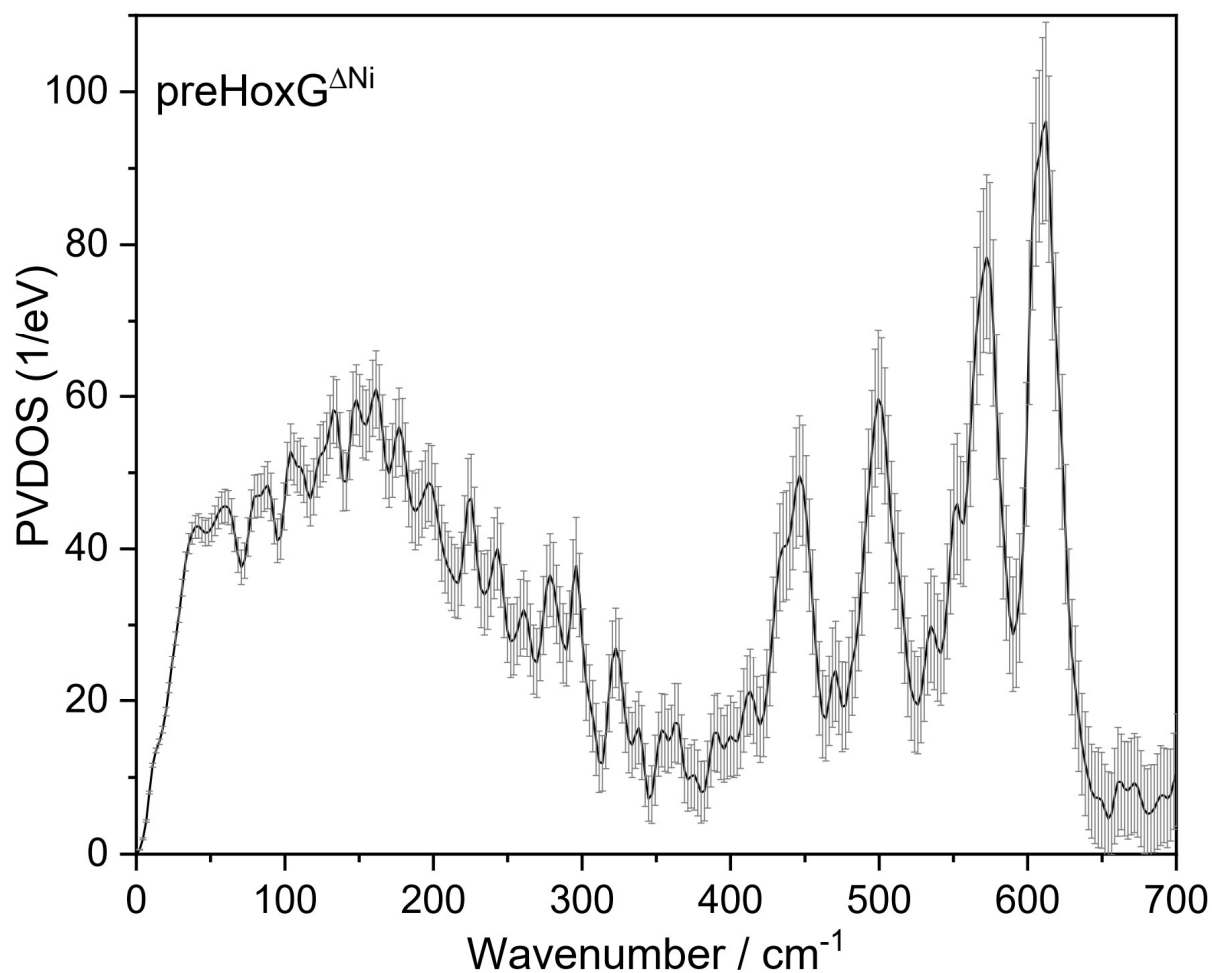
Supplementary Fig. 2: Infrared spectra of preHoxG proteins containing a Strep-tag II sequence either at the N-terminus (red trace, $^S\text{preHoxG}$) or the C-terminus (black trace, preHoxG^S) of the protein sequence. A single CO band is detected at 1953 cm^{-1} , while CN-related absorptions are observed at 2066 , 2076 and 2090 cm^{-1} . Comparison of the integrated areas of the CO bands of the two proteins suggests that preHoxG^S is loaded with only ca. 10 % of the $\text{NiFe}(\text{CN})_2(\text{CO})$ cofactor, in line with a previous substoichiometric Ni determination in preHoxG^S .⁸ The spectra are baseline-corrected and normalized to the protein-related amide II band at 1548 cm^{-1} . The IR experiments were performed with three independent samples and yielded consistent results ($n=3$).



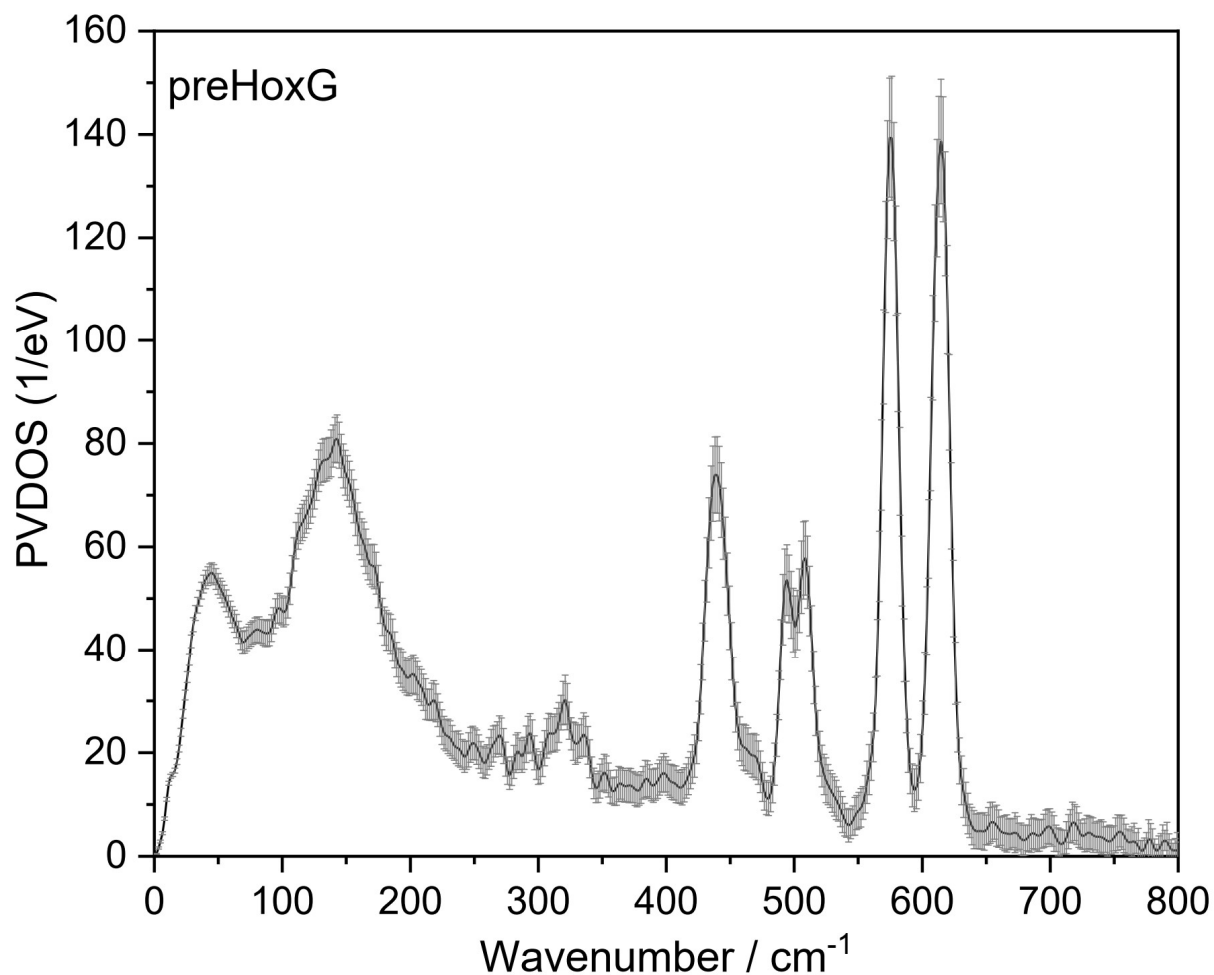
Supplementary Fig. 3: Comparison of the infrared spectra of mHoxG with procHoxG and preHoxG^{ΔNi} with procHoxG^{ΔNi}. 2nd derivative (a) and baseline-corrected (b) IR spectra of the mHoxG protein in comparison to those of the procHoxG analogue genetically devoid of the C-terminal peptide extension after normalization to the amide II band intensity at 1548 cm⁻¹. For a better illustration of the band composition, the absorbance spectra (b) are depicted as 2nd derivatives (a). For example, the band at 1958 cm⁻¹ (characteristic for Ni_i-B') can be clearly detected in the 2nd derivative of the spectrum (peak label in blue), while in the corresponding absorbance data it almost hidden by the main band (**Fig. 2c** and **Supplementary Fig. 3b**). 2nd derivative (c) and baseline-corrected (d) IR spectra of preHoxG^{ΔNi} protein in comparison to those of the procHoxG^{ΔNi} analogue genetically devoid of the C-terminal peptide extension after normalization to the amide II band intensity at 1548 cm⁻¹. The IR experiments were performed with three independent samples and yielded consistent results (n=3).



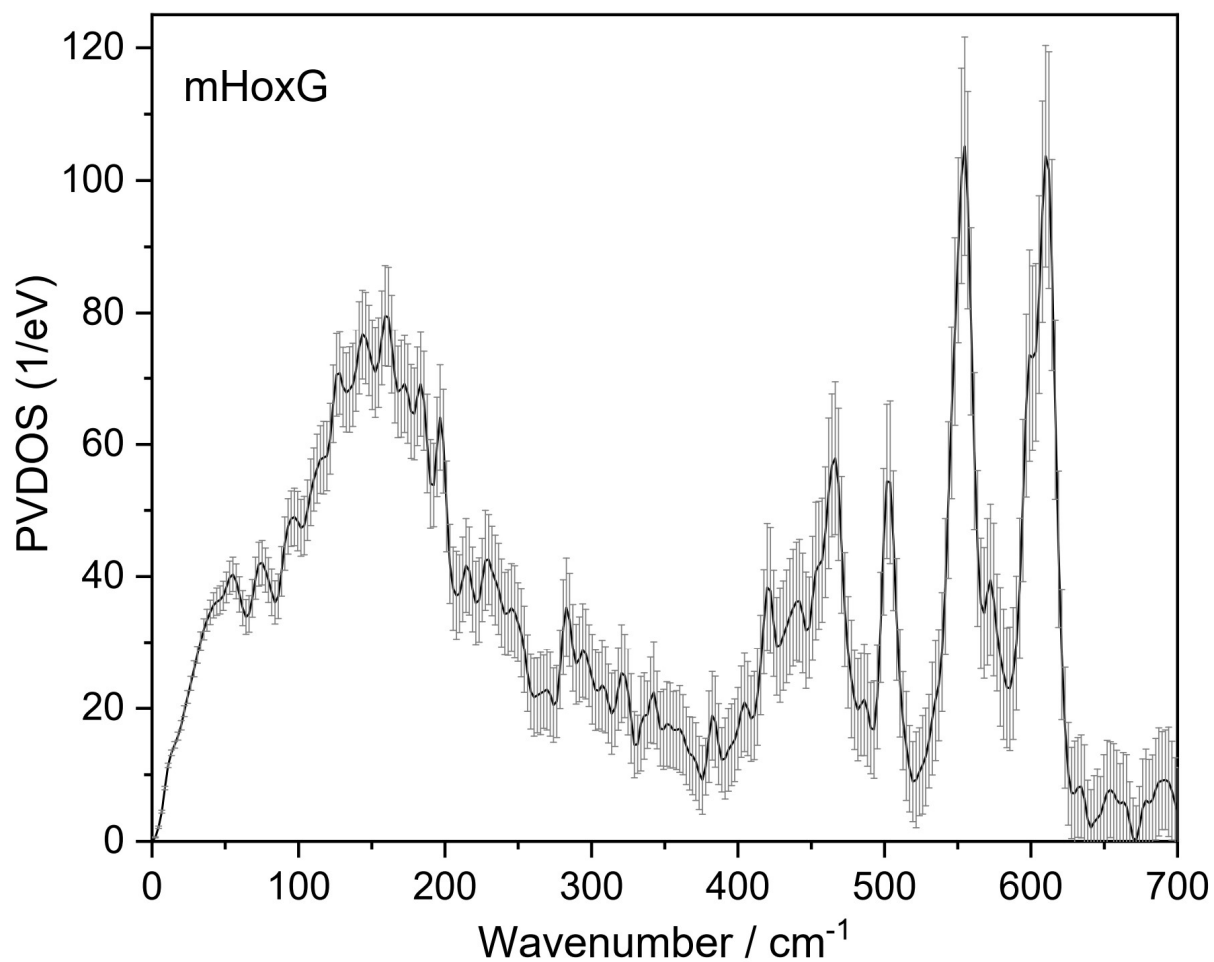
Supplementary Fig. 4: EPR spectra of preHoxG Δ FeNi, preHoxG Δ Ni, preHoxG and mHoxG. The spectra were recorded at 10 K with a microwave power of 1 mW. The spectra are normalized to the protein amount. All four intermediates show substoichiometric signals of high- and low-spin Fe³⁺ (red and cyan bars). These signals are most likely unrelated to the hydrogenase catalytic center, considering that the highest (signals) intensity was observed for preHoxG Δ FeNi, which is equipped with negligible amounts of CO and CN ligands (**Fig. 2c** and **Supplementary Fig. 1**). Additionally, in preHoxG Δ FeNi a minor axial species at a g-value of g = 2.01 has been tentatively attributed to traces of a [3Fe-4S] cluster (highlighted in green) corresponding to less than 5% of the entire spectrum. Finally, a broad rhombic signal of Ni_r-B' (blue bar) was detected in mHoxG, which is described in more detail in **Supplementary Fig. 11**.



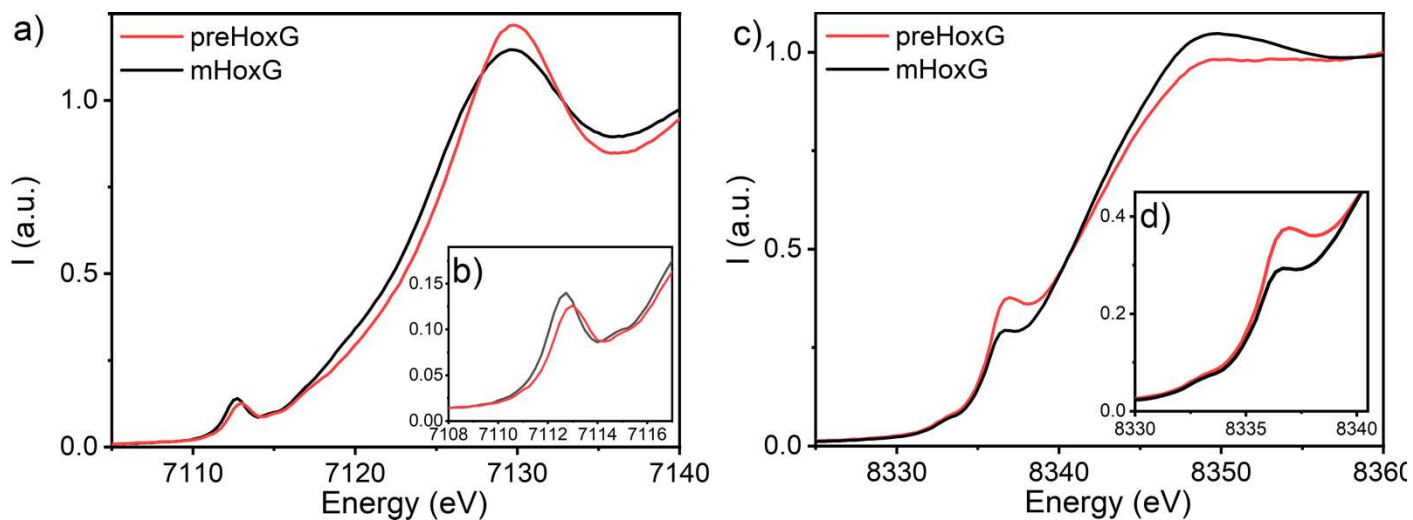
Supplementary Fig. 5: NRVs data of preHoxG^{ΔNi} including error bars. The black trace represents the experimental NRVs data (n=1) and the grey lines the corresponding error bars acquired at the beamline BL09XU of SPring-8. Details about the scanning parameters are reported in the methods section. See main text, for a detailed peak assignment.



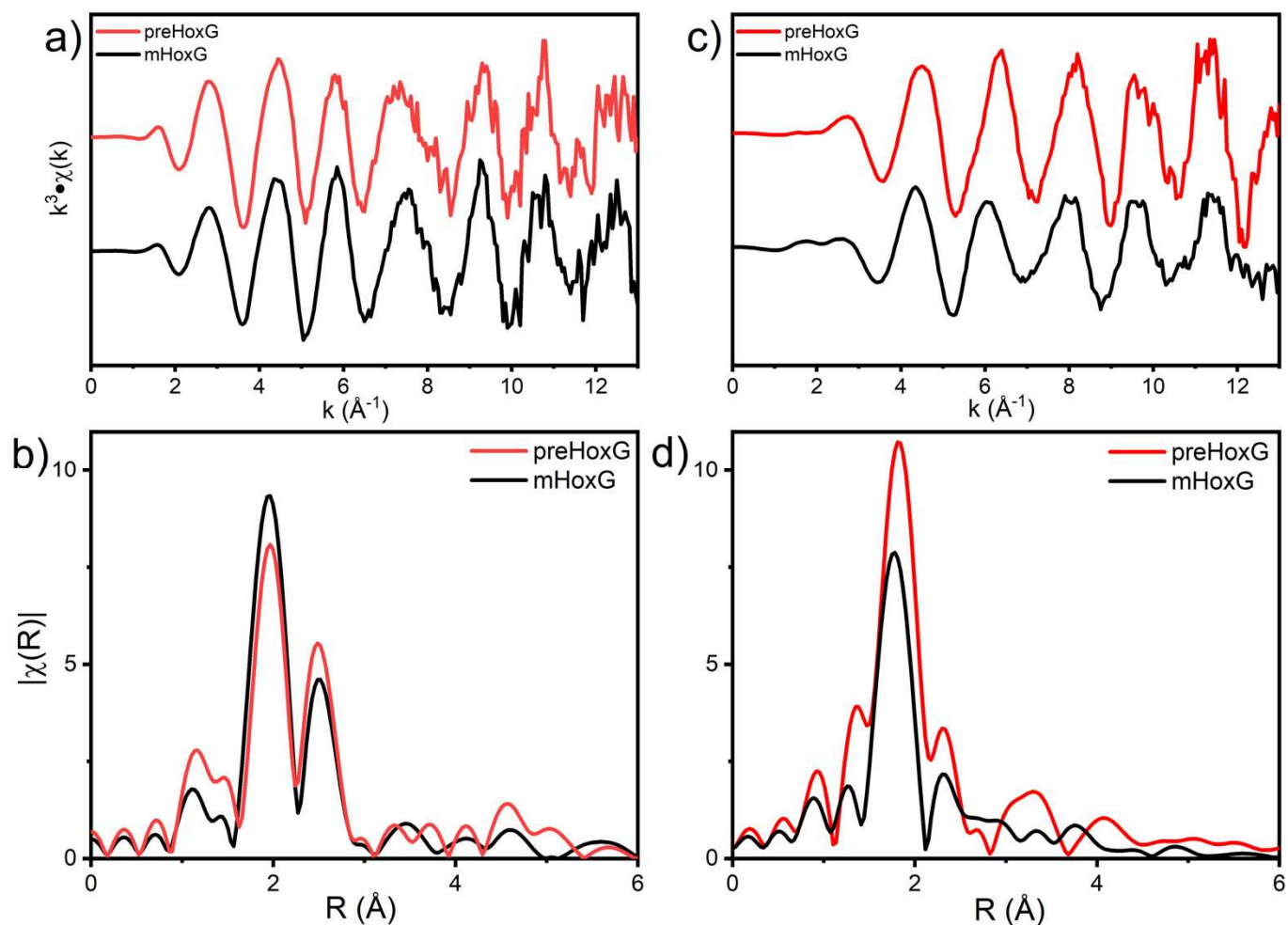
Supplementary Fig. 6: NRVs data of preHoxG including error bars. The black trace represents the experimental NRVs data ($n=1$) and the grey lines the corresponding error bars acquired at the beamline P01 of Petra III. Details about the scanning parameters are reported in the methods section. See main text, for a detailed peak assignment.



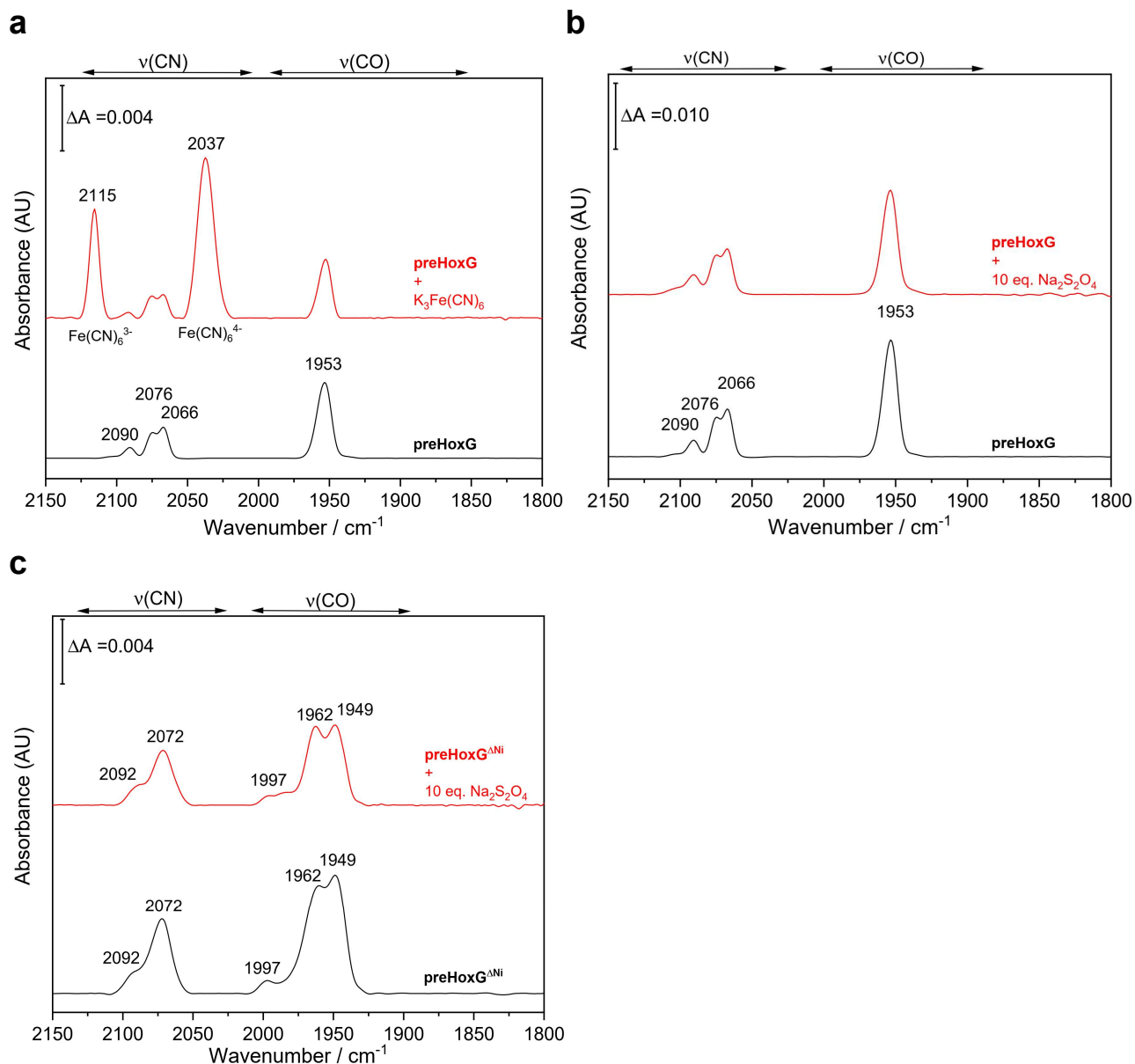
Supplementary Fig. 7: NRVS data of mHoxG including error bars. The black trace represents the experimental NRVS data (n=1) and the grey lines the corresponding error bars acquired at the beamline BL09XU of SPring-8. Details about the scanning parameters are reported in the methods section. See main text, for a detailed peak assignment.



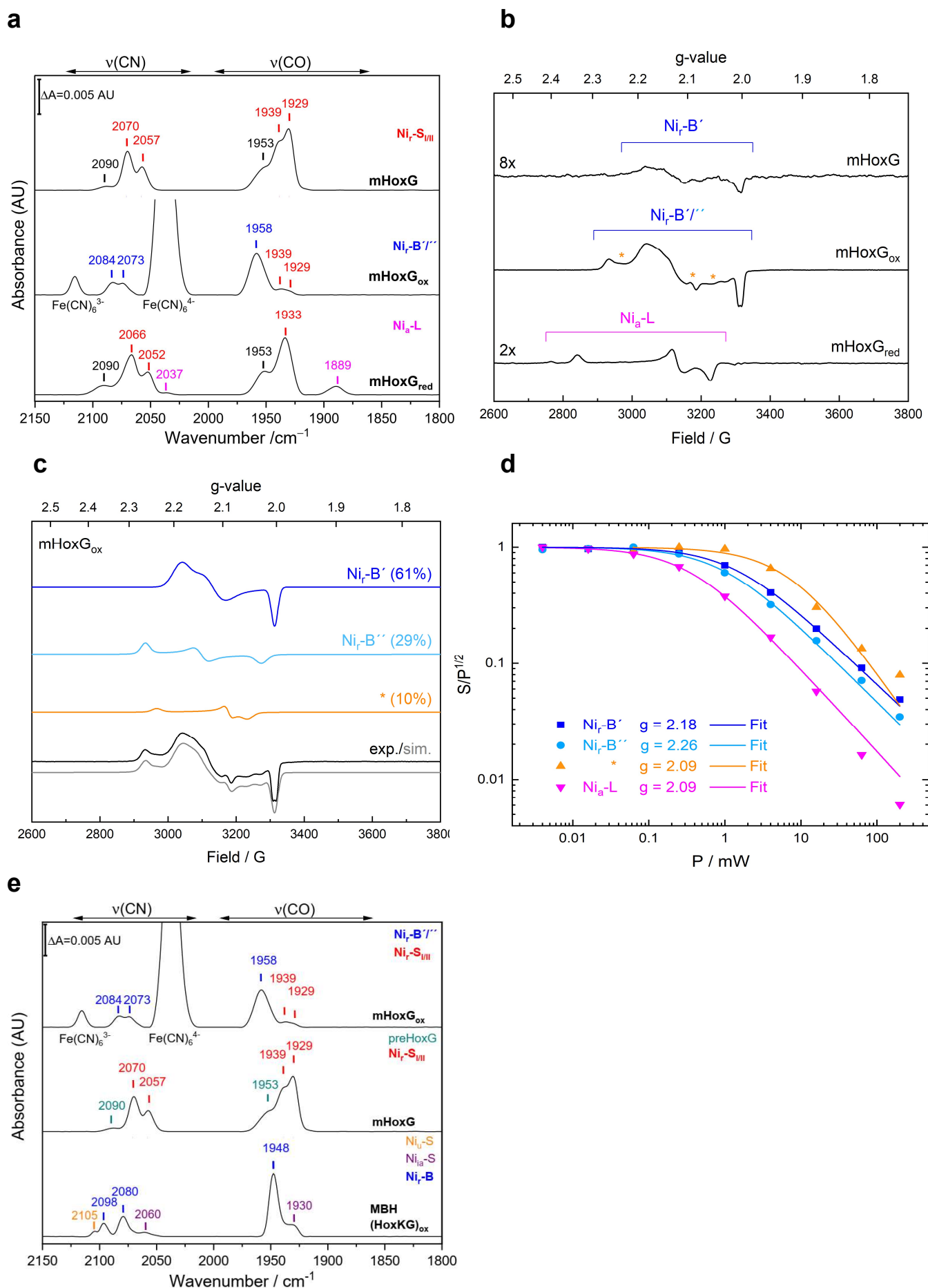
Supplementary Fig. 8: Normalized Fe K-edge (a, b) and Ni K-edge (c, d) XAS comparisons of preHoxG (red) and mHoxG (black). The provided inset highlights the pre-edge region.



Supplementary Fig. 9: Comparisons of Fe K-edge (a, b) and Ni K-edge (c, d) EXAFS of preHoxG (red) and mHoxG (black) in k-space (a, c) and R-space (b, d). R-space spectra were generated by transformation across a k-range of 3-12 $\text{\AA}^{-1}$.



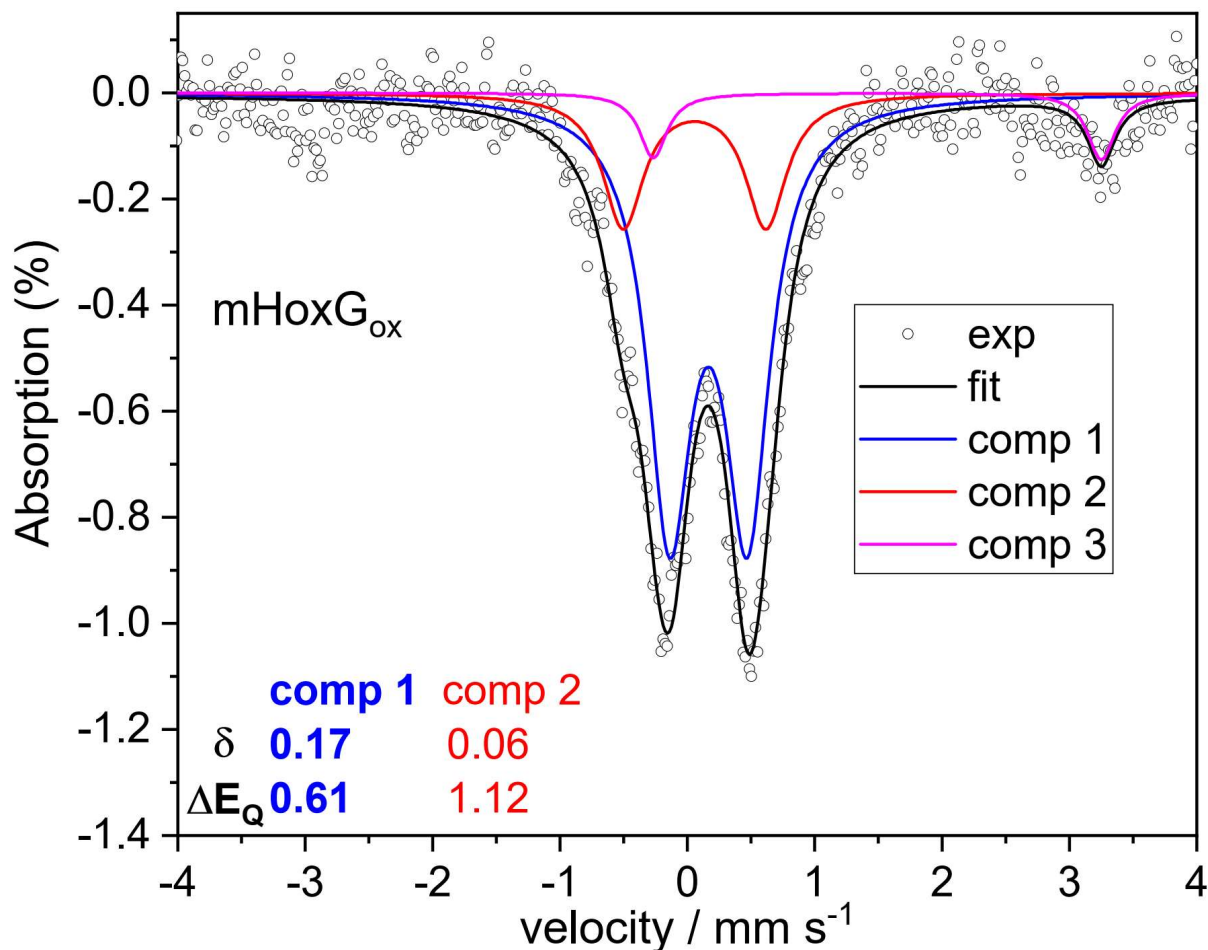
Supplementary Fig. 10: IR spectra of preHoxG and preHoxG^{ΔNi} before and after treatment with ferricyanide, $\text{K}_3\text{Fe}(\text{CN})_6$, and sodium dithionite, $\text{Na}_2\text{S}_2\text{O}_4$. **a)** Infrared spectra of preHoxG in its as-isolated form (black trace) and incubated with three equivalents of $\text{K}_3\text{Fe}(\text{CN})_6$ (red trace). The main CO and CN absorptions observed for both protein samples are located at 1953, 2066, 2076 and 2090 cm^{-1} , respectively. The absence of significant spectral changes of the active site bands reflects no change in the oxidation state of the [NiFe] cofactor. The two additional bands at 2037 and 2115 cm^{-1} correspond to the CN stretching modes of $\text{Fe}(\text{CN})_6^{3-}$ and $\text{Fe}(\text{CN})_6^{4-}$ species, respectively.⁹ **b)** IR spectra of preHoxG in the as-isolated form (black trace) and incubated with ten equivalents of sodium dithionite (red trace). The main CO and CN absorptions of both protein samples remain at 1953, 2066, 2076 and 2090 cm^{-1} , respectively. The absence of detectable spectral changes in the CO and CN stretching modes reflects again no change in the oxidation state of the [NiFe] active site, which remained in a diamagnetic $\text{Ni}^{\text{II}}\text{--Fe}^{\text{II}}$ configuration (**Supplementary Fig. 4**). **c)** IR spectra of preHoxG^{ΔNi} in its as-isolated form (black trace) and incubated with ten equivalents of $\text{Na}_2\text{S}_2\text{O}_4$ (red trace). The predominant CO and CN modes in both protein samples are detected at 1949 cm^{-1} , 1962 cm^{-1} , 1997 cm^{-1} , 2072 cm^{-1} and 2092 cm^{-1} , respectively. The absence of any detectable spectral changes reflects an unchanged oxidation state of the iron ion. The IR experiments were performed with three independent samples and yielded consistent results ($n=3$).



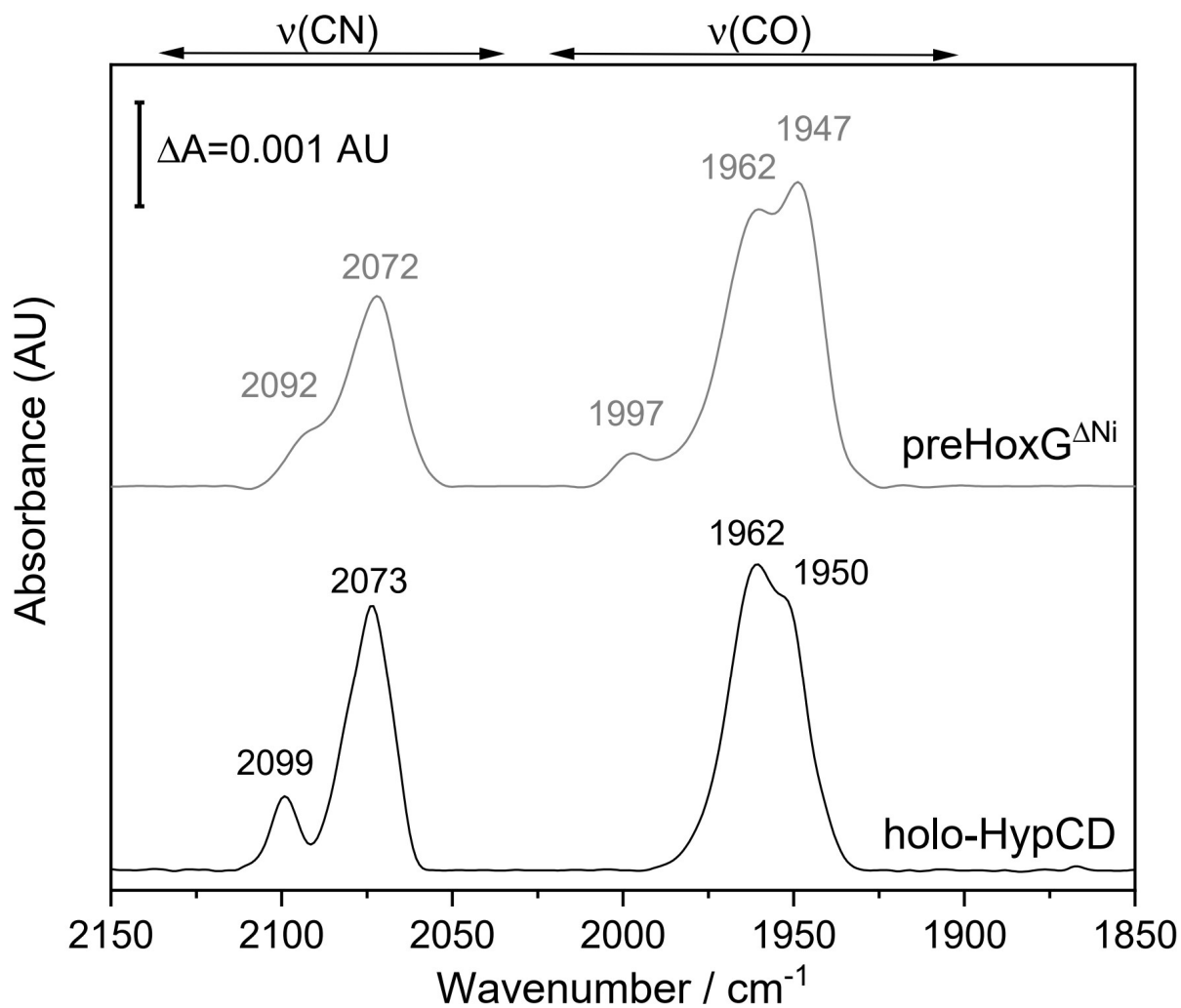
Supplementary Fig. 11: Spectroscopic data confirming redox reactivity in mHoxG and comparison of IR spectra of mHoxG and assembled MBH (HoxKG).(continued on the next page)

Supplementary Figure 11: continued.

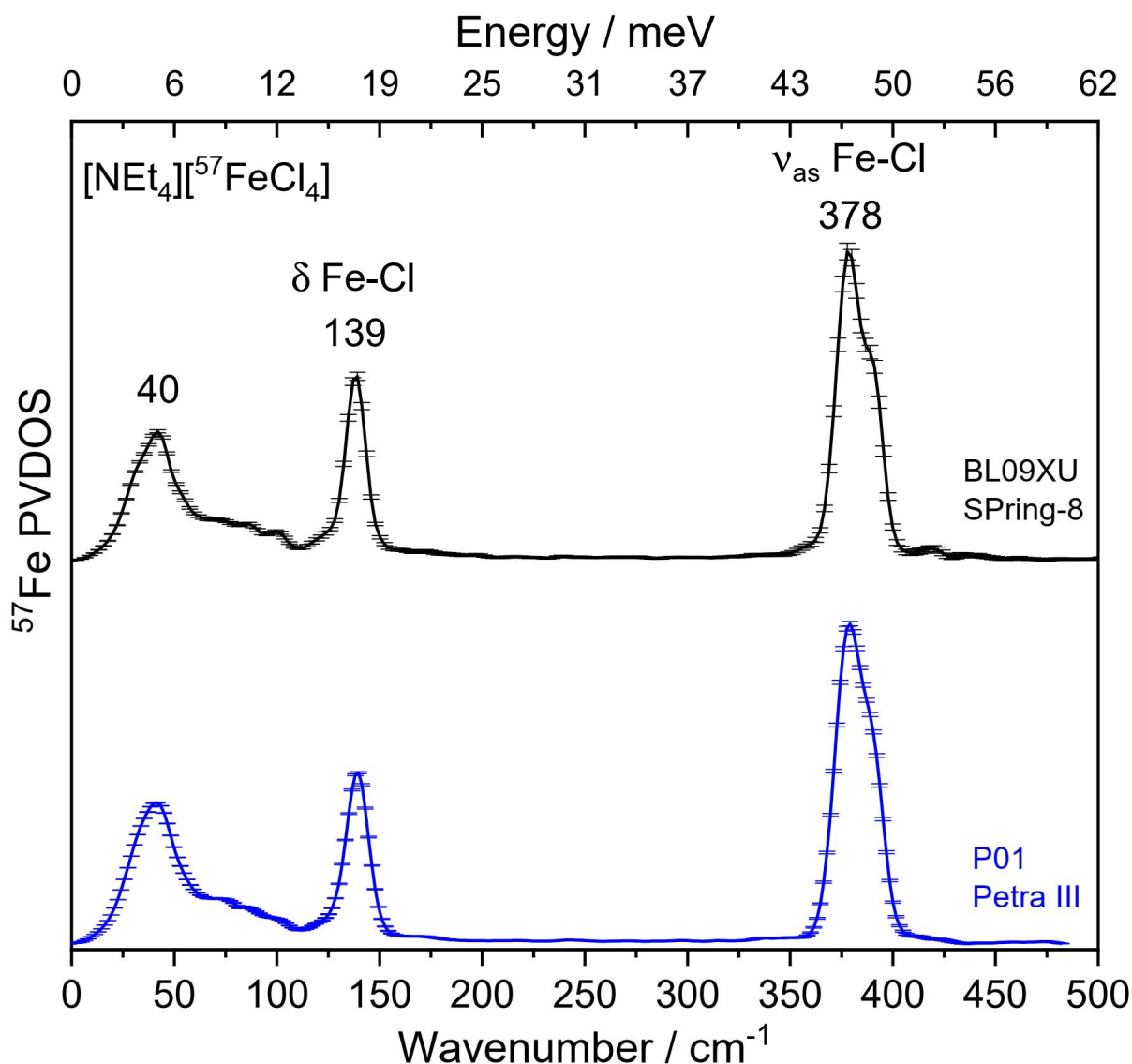
(a) Baseline-corrected IR spectra of as-isolated (top), ferricyanide-oxidized (middle) and Na₂S₂O₄-reduced (bottom) mHoxG. Absorptions or band patterns related to certain redox states of the active site are marked with the following color code: red, Ni_r-S_{I/II}; magenta, Ni_a-L; blue/light blue Ni_r-B^{'''}. Characteristic IR bands are labeled with the corresponding wavenumbers (cm⁻¹) and the respective redox state. Ferricyanide-treated mHoxG_{ox} shows two additional bands at 2037 and 2115 cm⁻¹, which correspond to the CN stretching bands of Fe(CN)₆³⁻ and Fe(CN)₆⁴⁻ species, respectively. The orange asterisks in (b) label an unknown EPR species ($g_1 = 2.239$, $g_2 = 2.090$, $g_3 = 2.053$). A similar signal has been observed in the air-oxidized [NiFe]-hydrogenase of *Desulfovibrio vulgaris* Miyazaki F and supposed to be non-physiological.¹⁰ (c) Deconvolution of the complex EPR spectrum of mHoxG_{ox} based on simulation of the individual components. (d) Power-dependent saturation of the different paramagnetic species of mHoxG_{ox} and mHoxG_{red}. The data was fitted to the empirical equation $S/P^{1/2} = 1 / ((1 + P/P_{1/2})^{b/2})$, where $P_{1/2}$ is the power of half-saturation and b is a parameter for the homogeneity of the line broadening.¹¹ The g -values indicate the field position for of the intensity (S) measurements. As previously observed for *C. necator* HoxC,⁹ the power saturation of Ni_r-B' and Ni_r-B'' is almost identical, indicating a similar electronic environment. (e) Baseline-corrected IR spectra of as-isolated (ferricyanide-oxidized) MBH_{ox} (bottom trace), as-isolated mHoxG (middle trace) and ferricyanide-treated mHoxG_{ox}. Absorptions related to specific redox states of the active site are marked with the following color code: red, Ni_r-S_{I/II}; purple, Ni_{ia}-S; orange, Ni_u-S; petrol, preHoxG; blue, Ni_r-B^{'''}. The main CO/CN stretching vibrations in MBH_{ox} (Ni_r-B, Ni^{III}) are blue-shifted compared with those in mHoxG (Ni_r-S_{I/II}, Ni^{II}), indicating a more oxidized cofactor in the MBH. Spectral differences between MBH and mHoxG are evident even at the same oxidation level (Ni_r-B),¹² reflecting the importance of the small subunit in fine tuning the electronic and molecular structure of the catalytic center. The spectra are normalized with respect to the amide II band intensity. The IR experiments were performed with three independent samples and yielded consistent results (n=3).



Supplementary Fig. 12: Mössbauer spectrum of ^{57}Fe -labeled mHoxG treated with $\text{K}_3\text{Fe}(\text{CN})_6$. The experimental and the simulated traces are represented in black. The components contribution to the simulated spectra are highlighted in blue (main component) and red (minor component). Adventitiously bound high-spin Fe^{II} contributions (comp 3) are represented in magenta. Isomer shift (δ) and quadrupole splitting (ΔQ) parameters are shown the graph and listed in **Supplementary Table 3**.



Supplementary Fig. 13: IR spectra recorded for preHoxG^{ΔNi} (grey trace) and holo-HypCD (black trace) in their as-isolated forms. The labeled bands are related to the CO and CN stretching vibrations of the diatomic active site ligands. The similarity of the band positions in the two IR spectra suggests a similar coordination for the Fe^{II}(CN)₂(CO) fragment in both proteins. The IR experiments were performed with three independent samples and yielded consistent results (n=3).



Supplementary Fig. 14: Calibrated nuclear resonance vibrational spectra of [NEt₄][⁵⁷FeCl₄] compound collected at P01 Petra III (blue trace, n=1) and BL09XU SPring-8 (black trace, n=1). Main peaks at 139 (17.2) and 378 cm⁻¹ (46.9 meV) are attributed to the Cl-Fe-Cl bending (δ) and the asymmetric Fe-Cl stretching (ν_{as}) modes respectively.¹³ The low energy intensity band at ca 40 cm⁻¹ (ca 5 meV) is related to FeCl₄⁻ acoustic and optical lattice modes.¹⁴ Data include error bars.

Supplementary Note

Detailed spectroscopic analysis of the HoxG intermediates

Comparison of the IR spectra in the processed and unprocessed HoxG intermediates

The positions of the dominating CO/CN bands at 1929, 1939, 2057 and 2070 cm^{-1} are conserved (**Supplementary Fig. 3**) in both mHoxG and procHoxG intermediates, confirming that the C-terminal extension in MBH is not required for the active site incorporation but significantly improves metal incorporation. In agreement with previous *in vivo* and *in vitro* findings,¹⁵ the cofactor loading of the “genetically processed” procHoxG^{ΔNi} and procHoxG proteins was much lower than that observed for their native counterparts preHoxG^{ΔNi} and mHoxG (**Supplementary Fig. 3**). In fact, the cofactor loading in procHoxG estimated via integration of the active site signals is about half of that in mHoxG. The discrepancy of active site loading can be attributed to the fact that the incorporation of the metal cofactor into the protein is compromised by the absence of the C-terminal extension. These data correlate with previous observations of reduced hydrogenase content in the cytoplasmic membrane when a genetic construct of MBH devoid of its C-terminal extension was used for protein synthesis.¹⁵ The CO absorption bands at 1953 and 1958 cm^{-1} in mHoxG (**Supplementary Figs. 3a, b**) can be attributed to a fraction of the protein sample still containing the C-terminal extension (preHoxG, 15-20%) and a minor population of mHoxG (< 5 %) equipped with an oxidized $\text{Ni}^{\text{III}}(\text{OH})\text{Fe}(\text{CN})_2(\text{CO})$ cofactor (**Supplementary Fig. 11a**). This correlates with the EPR data in **Supplementary Fig. 11b**, suggesting the presence of minor amounts of Ni^{III} species in as-isolated mHoxG. The shoulder of the CO band at 1950 cm^{-1} in procHoxG is most likely obscured in the spectrum of the mHoxG sample by the broad absorption at 1953 cm^{-1} of unprocessed preHoxG (**Supplementary Fig. 3b**).

Comparing the IR spectra of preHoxG^{ΔNi} and procHoxG^{ΔNi} analogue (**Supplementary Figs. 3c, d**), CO and CN bands are observed for both protein intermediates. This again confirmed that the C-terminal extension in MBH is not essential for the active site incorporation. Notably, the cofactor loading in procHoxG^{ΔNi}, estimated via the integration of the active site signals, appears to be about two thirds of that in preHoxG^{ΔNi} (**Supplementary Fig. 3d**). The lower active site loading in procHoxG^{ΔNi} is consistent with the observations for procHoxG, indicating that the cofactor insertion is hampered. The CO absorption at 1962 cm^{-1} in preHoxG^{ΔNi} is absent from the IR spectrum of procHoxG^{ΔNi} (**Supplementary Fig. 3c, d**), in which an additional CO band is detected at 1975 cm^{-1} . This might indicate an alternative coordination of the inorganic cofactor in preHoxG^{ΔNi} due to the presence of the C-terminal extension.

Detailed XAS analysis of the preHoxG and mHoxG intermediates

X-ray absorption spectroscopy has previously been used to investigate the nature and binding lengths of atoms near the NiFe site of [NiFe]-hydrogenases.^{16,17} The Fe and Ni K-edge XAS measurements were performed for the preHoxG and mHoxG intermediates. The Fe K-edge XAS comparison of preHoxG, and HoxG is provided in **Supplementary Fig. 8**. Both spectra appear unique. The edge position of the two HoxG variants is dominated by the coordination environment due to the presence of highly covalent CN^- and CO ligands, where a larger proportion of heavier coordinating atoms (S) result in a lower energy edge position. PreHoxG has the highest energy edge (7117-7125 eV) combined with the most intense white line (~7130 eV). The edge position shifts by -0.8 eV moving to mHoxG. PreHoxG also has the least intense pre-edge (7113 eV). The pre-edge region arises from $1s \rightarrow 3d$ excitations, and the intensity of the Fe K-edge pre-edge is dependent upon both 3d-

4p mixing (i.e., local Fe site symmetry) and oxidation state. With lower symmetry, greater 3d-4p mixing arises producing greater pre-edge intensity. Additionally, a small feature 7114.5 eV is observed, which may arise from a Fe 1s Ni 3d or MLCT transition. For mHoxG, an edge shift of -0.8 eV is observed.

Looking to the Fe K-edge EXAFS in k-space (**Supplementary Fig. 9a**), the two samples are relatively similar, with the most noticeable differences appearing around $k = 8 \text{ \AA}^{-1}$.

Translating to R-space (**Supplementary Fig. 9b**), the samples exhibit three characteristic signals around $R \sim 1.3, 2, \text{ and } 2.5 \text{ \AA}$, which vary in relative intensity and position. The features at $R \sim 1.3, 2$ likely arise from the Fe–C and Fe–S scatterers, respectively, while the feature at $\sim 2.5 \text{ \AA}$ likely arises from a convolution of the Fe–Ni single scattering and Fe–C–N/O double scattering paths. The low-R Fe–C scatterer appears most intense in preHoxG and less intense in HoxG. Intensity is dependent both on degeneracy and distribution, the latter of which is a combination of the similarity of Fe–C scatterers which have been grouped together and throughout the sample bulk (static disorder), and thermal disorder. The Fe–S scatterer has higher intensity in mHoxG. The third feature at $R \sim 2.5 \text{ \AA}$ is most intense in preHoxG.

At the Ni K-edge, preHoxG displays a prominent feature in the rising edge at 8337.0 eV, along with a small shoulder to lower energy, $\sim 8333.2 \text{ eV}$ (**Supplementary Fig. 8c,d**). These likely arise from $1s \rightarrow 4p$ and $1s \rightarrow 3d$ transitions, respectively. The presence of a lower energy $1s \rightarrow 4p$ transition would be consistent with a geometry closer to square planar rather than tetrahedral,¹⁸ but arguably could arise from the presence/absence of a fifth ligand. Moving to mHoxG, the intensity of the primary pre-edge feature shifts to slightly lower energy at 8336.7 eV and decreases significantly in intensity. The pre-edge shoulder in preHoxG and mHoxG appear identical. Lastly, an increase in white line intensity ($\sim 8346\text{--}8352 \text{ eV}$) is observed for mHoxG. No significant change in edge position is observed, implying preHoxG and mHoxG are in the same oxidation state, consistent with the EPR data (**Supplementary Fig. 4**). However, the changes observed in the pre-edge and white line regions are indicative of a change in geometry and/or coordination.

Moving to the Ni EXAFS in k-space (**Supplementary Fig. 9c**), preHoxG and mHoxG present unique spectra. They differ significantly between $5\text{--}8 \text{ \AA}^{-1}$, and move into phase at higher k . In R-space (**Supplementary Fig. 9d**), these spectra produce a single dominant common feature $\sim 1.9 \text{ \AA}$, which is tentatively assigned as arising from a Ni–S scatterer. Additionally, higher R features are observed between $2.3\text{--}3.5 \text{ \AA}$ (varying between the systems) which may arise from Ni–Fe and/or long Ni–C _{γ} -Cys scatterers.

All models used to simulate the experimental data are provided in the **Supplementary Tables 4 and 5**. The bond lengths of the thiolates and the CO/CN ligands to the metal ions in the best-fitting models also do not differ significantly between the two HoxG intermediates, beyond the contraction of Ni–S2 in preHoxG to become equivalent with Ni–S1 and the expansion of Fe–C2 in mHoxG to make this scatterer undifferentiable from Fe–C1. Notably, unlike mHoxG, a split of the Fe–C scattering shell was necessary to obtain an appropriate model for preHoxG. In summary, the spectra of preHoxG and mHoxG support the assignment of oxidation states Fe(II) and Ni(II) in agreement with the EPR and Mössbauer data (**Fig. 3a,b** and

Supplementary Fig. 4). EXAFS results imply that Fe is 2-Cys coordinated in both preHoxG and mHoxG. Conversion of preHoxG to mHoxG appears to involve the incorporation of a hydroxy (OH^-) ligand into the active site. This involves additional geometrical changes, including a lengthening of the Fe-Ni distance, lengthening of a Ni-S bond, and a change in Ni geometry resulting in longer Ni-C (Cys) distances and a decrease in energy of the $1s \rightarrow 4p$ transition. It should be noted that the Ni K-edge data of mHoxG revealed a Ni-O scatterer with an occupancy between 0.5 and 1.0 (**Fig. 5b**). Cross-checking this reduced occupancy at the Fe edge reveals no improvement in fit, suggesting a mixture of species in which about half would have a bridging Fe-OH-Ni and the other half a terminal Fe-OH. The presence of several species in mHoxG was also evidenced by NRVs, IR, and Mössbauer spectroscopies, which showed the presence of about 20% preHoxG in the mHoxG samples (**Figs. 2c, 3a, and 4a**).

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