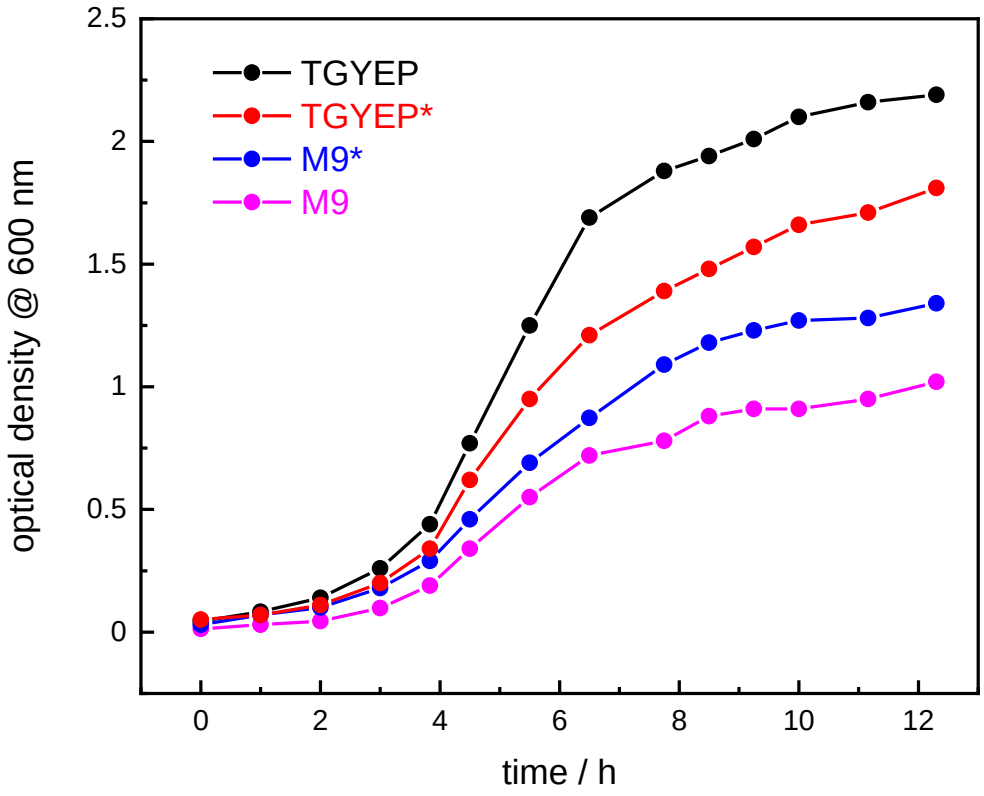

Figure S1. *E. coli* growth curve in different cultivation media. Large scale protein purification was performed with 20 liters of anaerobic TGYEP full medium (black trace) at 37 °C with a typical yield of 3–4 g of cells/liter. To produce the ^{57}Fe-labeled HypCD complex for Mössbauer spectroscopy, the bacteria were grown anaerobically either in modified minimal medium (M9*, blue trace) or modified full medium (TGYEP*, red trace).

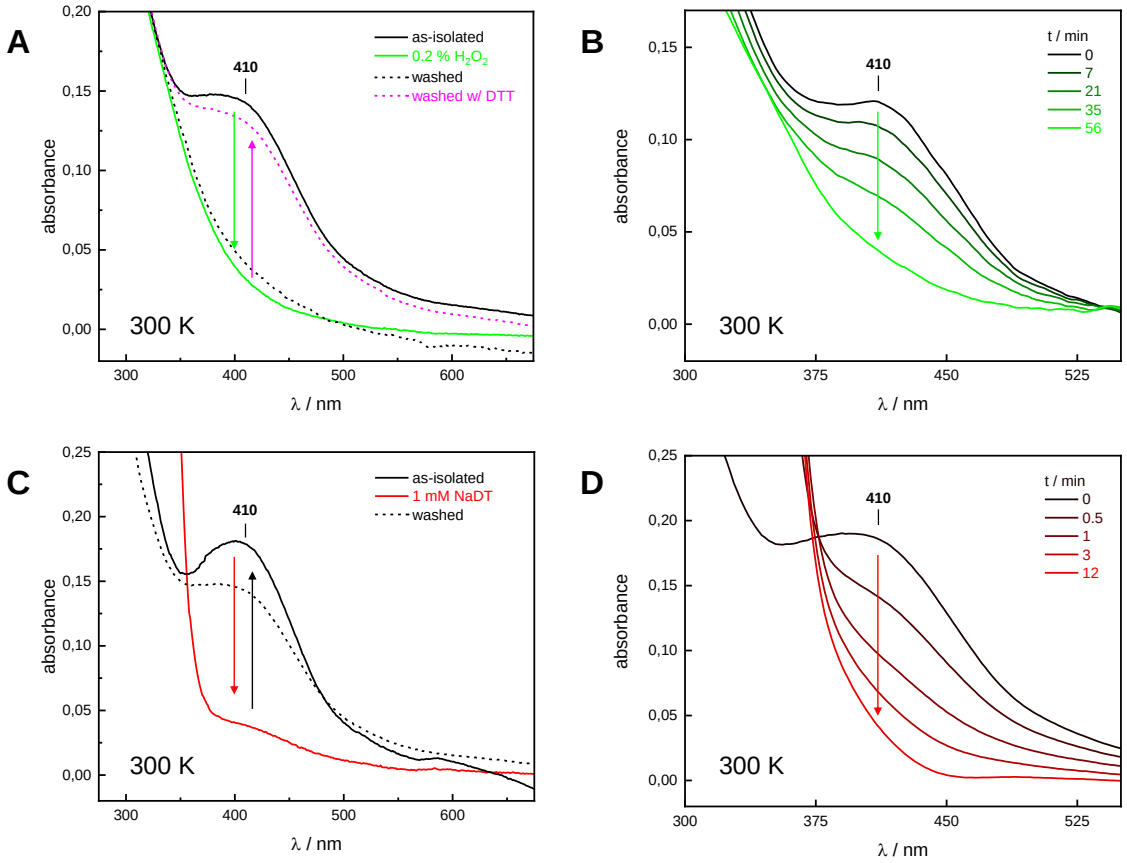

Figure S2. UV/Vis spectroscopy. Protein solution with 1–2 mg/ml HypCD was prepared anaerobically and probed in a septum-sealed quartz cuvette. The reaction was started in situ upon injection of NaDT or H_2O_2 stock solution. **(A)** Disappearance of the band at 410 nm upon oxidation with 0.2 % H_2O_2. After washing (buffer exchange), no restoration of the signal was observed (black dashed traces). Mild reduction of the washed sample with DTT restored the band at 410 nm (magenta dashed trace). **(B)** Disappearance of the band at 410 nm upon oxidation followed over time. **(C)** Disappearance of the band at 410 nm upon oxidation with 1 mM NaDT. A simple buffer exchange largely restored the band at 410 nm (black dashed trace). **(D)** Disappearance of the band at 410 nm upon reduction followed over time.

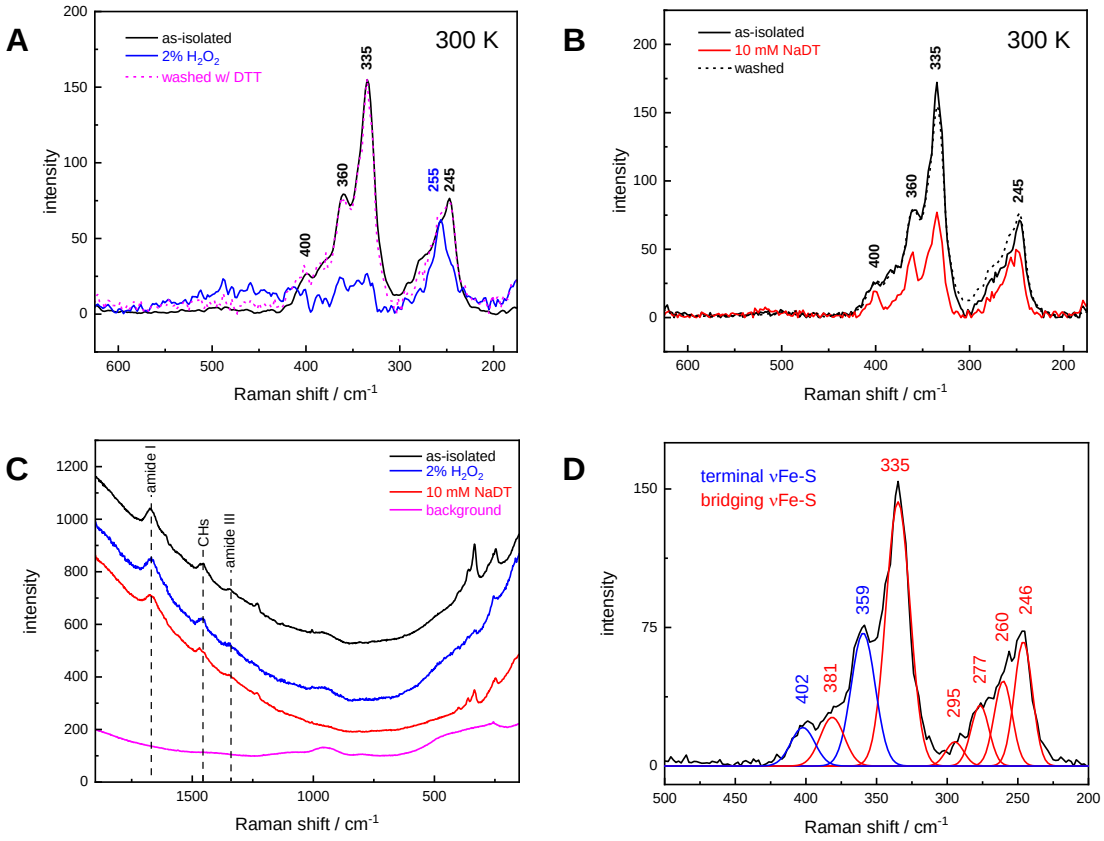

Figure S3. Resonance Raman spectroscopy. (A) Disappearance of the Raman signature upon oxidation with 2 % H_2O_2. After washing (buffer exchange), no restoration of the signal was observed (data not shown). Mild reduction of the washed sample with DTT restored the Raman bands (magenta dashed trace). **(B)** Depletion of the band at 410 nm upon oxidation with 10 mM NaDT. A simple buffer exchange largely restored the Raman signature (black dashed trace). **(C)** Resonance Raman raw data including HypCD in the as-isolated state (black), in the presence of 2 % H_2O_2 (blue), or with 10 mM NaDT (red) as well as scattering of the background (magenta). Protein marker bands at higher frequencies are marked. **(D)** Baseline-corrected Resonance Raman spectrum of HypCD in as-isolated form. The suggested components are in excellent agreement with the work of Spiro and colleagues.[1]

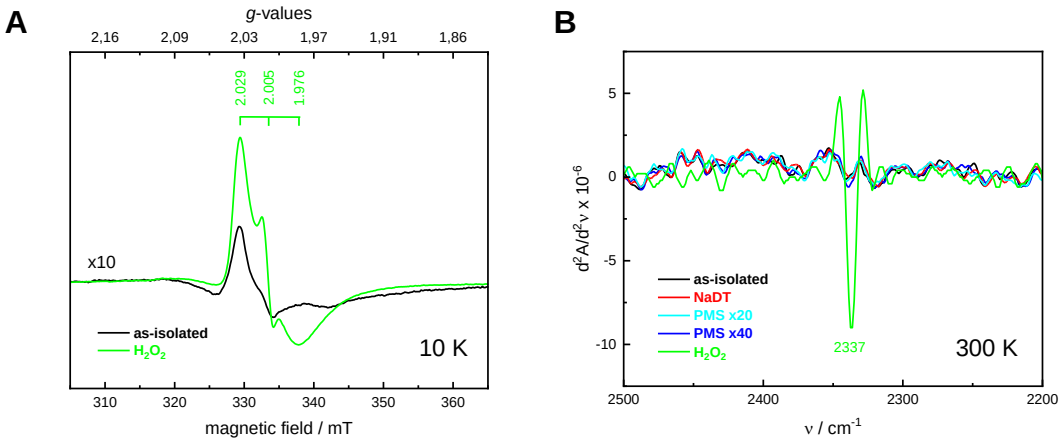

Figure S4. Oxidative damage in the presence of H_2O_2. **(A)** EPR spectra of HypCD (30–40 mg/ml) in the as-isolated state (black, diamagnetic) and in the presence of 2% H_2O_2 (green). The rhombic signal prominently observed upon oxidation with H_2O_2 has been assigned to a $[3Fe\text{-}4S]^+$ species of the iron-sulfur cluster previously.[2] Small traces of this species can be found in as-isolated preparations as well (x10 magnified for comparison). **(B)** Second derivative ATR FTIR spectra clearly show the formation of CO_2 in H_2O_2-incubated samples of HypCD. The indicative feature at 2337 cm^{-1} has been observed with HypCD before[3]; however, never upon incubation with NaDT or PMS.

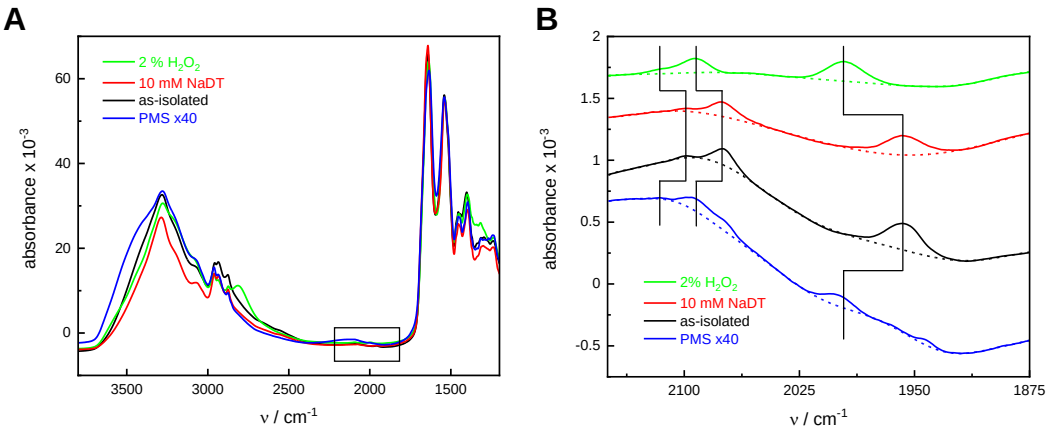

Figure S5. ATR FTIR absorbance spectra. **(A)** Raw data of ATR FTIR absorbance spectra of H_2O-hydrated films of HypCD in the as-isolated state (black), in the presence of H_2O_2 (green), NaDT (red), or PMS (blue). **(B)** Absorbance bands of the $[Fe](CN)_2CO$ cofactor precursor. Note the shift to higher frequencies in the presence of H_2O_2 (green) and PMS (blue). The dashed lines represent the background (the depicted frequency regime includes the broad 'combination' band of liquid H_2O).

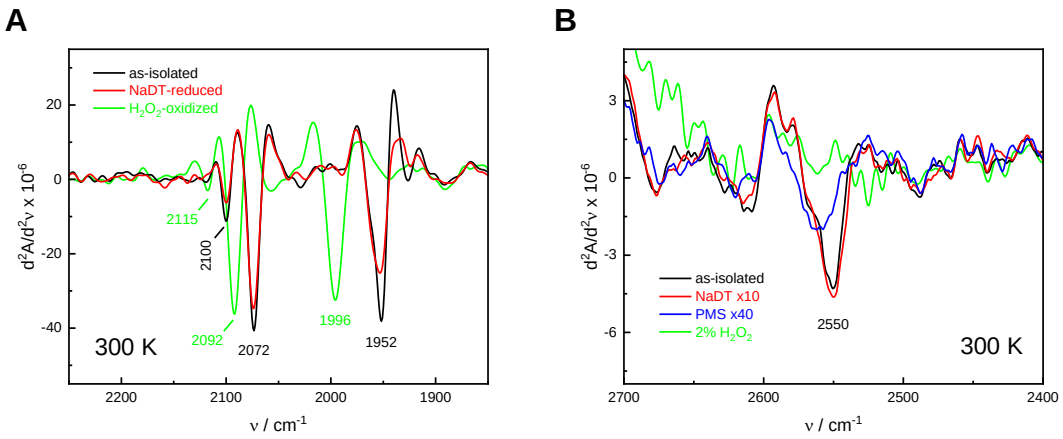

Figure S6. Oxidation of HypCD in the presence of H_2O_2. (A) Second derivative ATR FTIR spectra of the HypCD complex in as-isolated form (black), in the presence 2 % H_2O_2 (green), or restored with 10 mM NaDT after oxidation (red). Note the shift of the CO/CN^- bands to higher frequencies upon oxidation with H_2O_2. As-isolated and NaDT-restored samples show the same band positions. **(B)** As-isolated (black) and NaDT-reduced HypCD (red) shows a clear band at 2550 cm^{-1} that can be assigned to SH vibration of cysteine residues. In the presence of 40 eq. PMS or 2 % H_2O_2 the absorbance decreased (blue and green traces), indicative of disulfide bond formation.

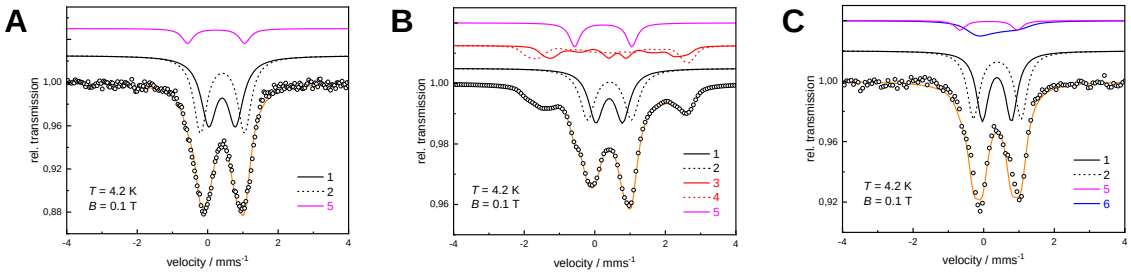

Figure S7. Mössbauer spectroscopy at weak *B*-fields. Spectra of the ^{57}Fe-labeled HypCD complex (40–50 mg/ml at pH 9) in as-isolated **(A)**, NaDT-reduced **(B)**, and PMS-oxidized form **(C)** obtained at 4.2 K and an external *B*-field or 0.1 T. Legend: experimental data (open circles), simulation (orange trace), $[4Fe\text{-}4S]^{2+}$ (1+2, black), $[4Fe\text{-}4S]^{+}$ (3+4, red), $[Fe]^{2+}$ (5, magenta), and $[Fe]^{3+}$ (6, blue).

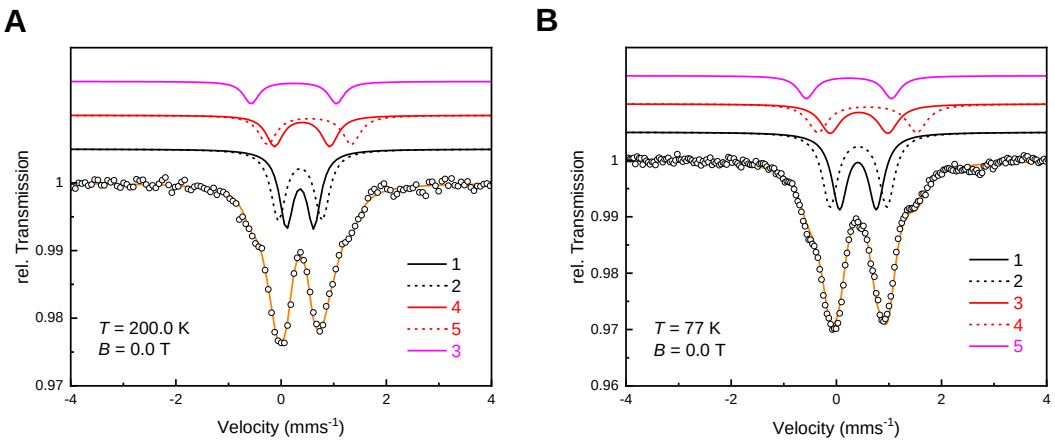

Figure S8. Temperature effects. Mössbauer spectra of the ^{57}Fe-labeled HypCD complex (40–50 mg/ml at pH 9) in reduced form obtained at 200 K **(A)** and at 77 K **(B)**. The respective parameters can be found in Table 2 in the main script. Legend: experimental data (open circles), simulation (orange trace), $[4Fe\text{-}4S]^{2+}$ (1+2, black), $[4Fe\text{-}4S]^{+}$ (3+4, red), and $[Fe]^{2+}$ (5, magenta).

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2. Blokesch, M. *et al.* The complex between hydrogenase-maturation proteins HypC and HypD is an intermediate in the supply of cyanide to the active site iron of [NiFe]-hydrogenases. *J. Mol. Biol.* **344**, 155–167 (2004).

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