Electronic Supplementary Material (ESI) for Dalton Transactions.

Supporting Information for

HydG, the "Dangler" Iron, and Catalytic Production of Free CO and CN^-: Implications for [FeFe]-Hydrogenase Maturation

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Table S1. Nomenclature and abbreviations used in this work

Abbreviation	Description
[2Fe]$_H$	2Fe subcluster of the H-cluster of HydA
[2Fe]$_F$	2Fe subcluster precursor assembled on HydF by HydE and HydG
[2Fe]$_{MIM}$	2Fe synthetic precursor clusters
HydA$^{\Delta EFG}$	HydA expressed in the absence of maturases, contains [4Fe-4S]$_H$ but no [2Fe]$_H$
HydA	[FeFe]-hydrogenase
HydG	Maturase that generates CO and CN^- H-cluster ligands from tyrosine
HydG$^{\Delta EF}$	HydG expressed in *E. coli* without co-expression of the other maturases
HydG^{EF}	HydG co-expressed with HydE and HydF and then purified away from them
HydG$^{\Delta CTD}$	HydG lacking the entire C-terminal domain and C-terminal [Fe-S] cluster
HydG^{H272A}	HydG variant used to probe the role of H272
HydE$^{\Delta GF}$	Maturase HydE expressed in the absence of HydG and HydF
HydF$^{\Delta EG}$	Maturase HydF expressed in the absence of HydE and HydG

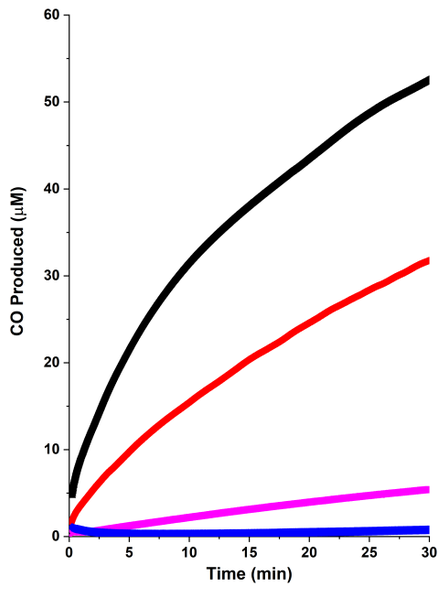

Figure S1. Buffer dependent HydG catalyzed CO formation kinetics, as monitored via binding to human deoxyhemoglobin. Single wavelength kinetics (A_{419nm}) were monitored for formation of carboxyHb, as previously reported.[1] Assays contained WT HydG$^{\Delta EF}$ (40 μM protein with 13.3 ± 0.2 Fe/protein), 1 mM L-tyrosine, 1 mM SAM, 5 mM NaDT, and 80 μM deoxyHb (per heme) at 37 °C. The spectral traces correspond to: black, 50 mM Tris, 10 mM KCl, pH 8.5; red, 50 mM HEPES, 10 mM KCl, pH 7.4; blue, 50 mM potassium phosphate, 10 mM KCl, pH 7.4; and magenta, 50 mM potassium phosphate, 10 mM KCl, pH 8.5. Human hemoglobin was kindly provided by Professor David Singel and Mr. David Schwab (Montana State University).

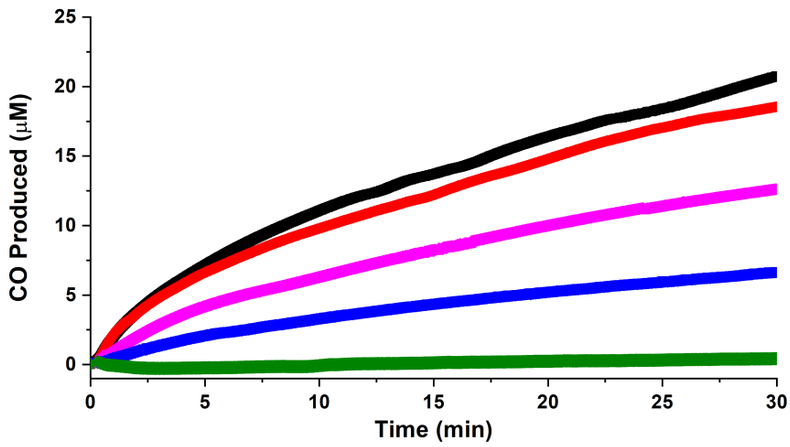

Figure S2. Effect of phosphate on WT HydG CO formation. Time course experiments monitoring the A_{419nm} increase associated with HbCO formation in the presence of varied phosphate concentrations. Assays contained WT HydG$^{\Delta EF}$ (51 μM protein with 5.6 ± 0.2 Fe/protein), 1 mM L-tyrosine, 1 mM SAM, 5 mM NaDT, and 100 μM deoxyHb (per heme) at 37 °C. The concentration of phosphate was varied using a mixture of Tris and phosphate buffers (250 mM Tris, 300 mM KCl, 5% glycerol, pH 8.5, and 250 mM potassium phosphate, 300 mM KCl, 5% glycerol, pH 8.5). The spectral traces correspond to: black, 230 mM Tris, no phosphate; red, 229 mM Tris, 1 mM phosphate; magenta, 221 mM Tris, 9 mM phosphate; blue, 207 mM Tris, 23 mM phosphate; and green, 5 mM Tris, 225 mM phosphate.

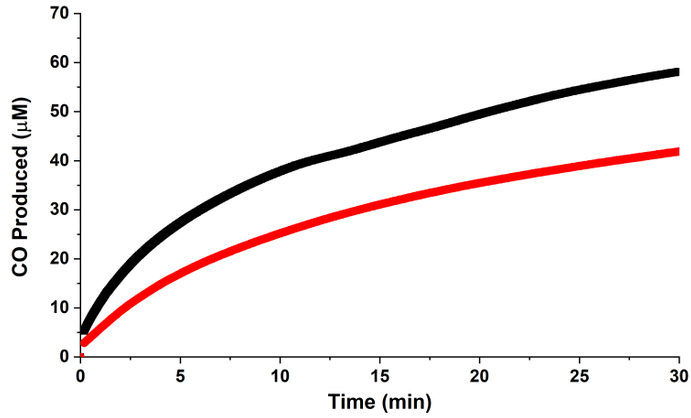

Figure S3. Comparison of WT HydG CO formation assays in the presence of reporting heme proteins H64L sperm whale myoglobin (black) and human hemoglobin (red). Assays were performed in 50 mM Tris, 10 mM KCl, pH 8.5 buffer and contained WT HydG$^{\Delta EF}$ (40 μM protein with 6.6 ± 0.7 Fe/protein), 1 mM L-tyrosine, 1 mM SAM, 1 mM NaDT, and either 81 μM deoxyHb or 76 μM H64L deoxyMb (per heme). Single wavelength kinetics at 419 nm (human Hb) or 425 nm (H64L Mb) were monitored at 37 °C to monitor formation of either carboxyHb or carboxyMb, respectively.

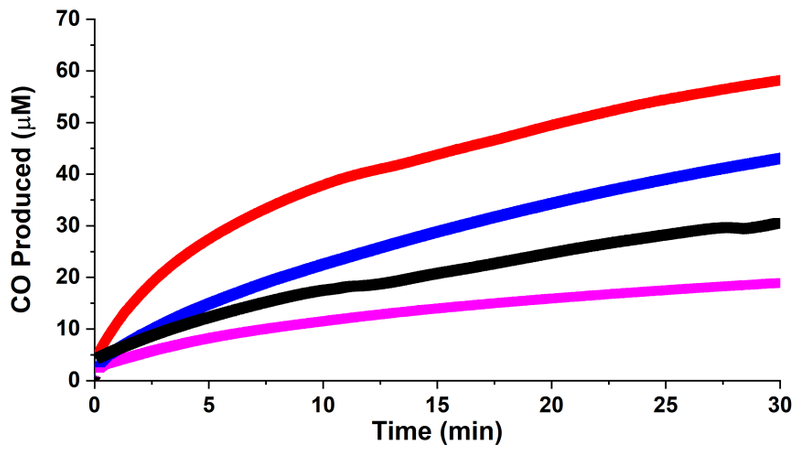

Figure S4. Effect of salt and glycerol on HydG catalyzed CO production assays. Assays contained WT HydG$^{\Delta EF}$ (40 μM protein with 6.6 ± 0.7 Fe/protein), 1 mM L-tyrosine, 1 mM SAM, 1 mM NaDT, and 80 μM H64L deoxyMb (per heme). Single wavelength kinetics at 425 nm were monitored at 37 °C to monitor formation of carboxyMb. The spectral traces correspond to: magenta, 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4; blue, 50 mM HEPES, 45 mM KCl, 0.2% glycerol, pH 7.4; black, 50 mM Tris, 300 mM KCl, 5% glycerol, pH 8.5; red, 50 mM Tris, 46 mM KCl, 0.2% glycerol, pH 8.5.

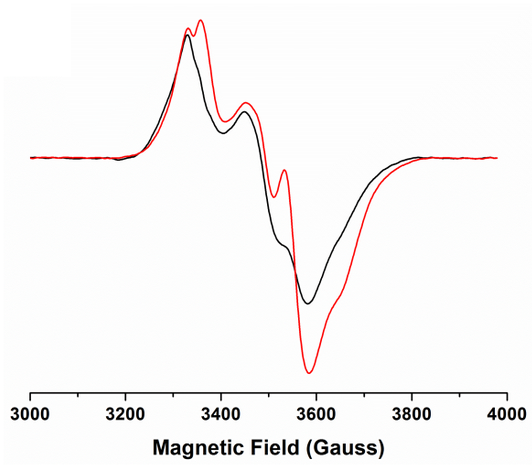

Figure S5. Low temperature (10.0 K, 1 mW), CW X-band EPR spectra of traditionally reconstituted HydG^{EF}. **A**. WT traditionally reconstituted HydG^{EF} (64 μM protein with 7.38 ± 0.40 Fe/protein) treated with 3 mM DT (black spectrum), and treated with 3 mM DT and 2 mM SAM (red spectrum).

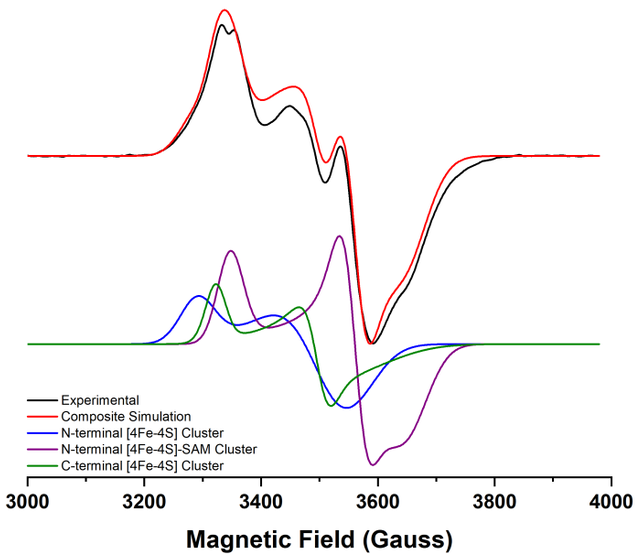

Figure S6. Simulations of the 10 K, 1 mW EPR data for dangler reconstituted HydG in the presence of 3 mM DT and 2 mM SAM (see experimental data in **Figure 3B**). Simulations were performed in EasySpin and results were plotted using Origin 2019b. The composite signal is shown as a combination of three spin systems, which are offset for clarity. See figure legend for explanation. The simulated g-values and g-strain follow: N-terminal [4Fe-4S] cluster (g = 2.034, 1.922, 1.887 with g_{strain} = 0.042, 0.064, 0.058); N-terminal [4Fe-4S] with SAM bound (g = 2.001, 1.881, 1.835 with g_{strain} = 0.031, 0.025, 0.044); C-terminal [4Fe-4S] cluster (g = 2.015, 1.913, 1.871 with g_{strain} = 0.023, 0.022, 0.088).

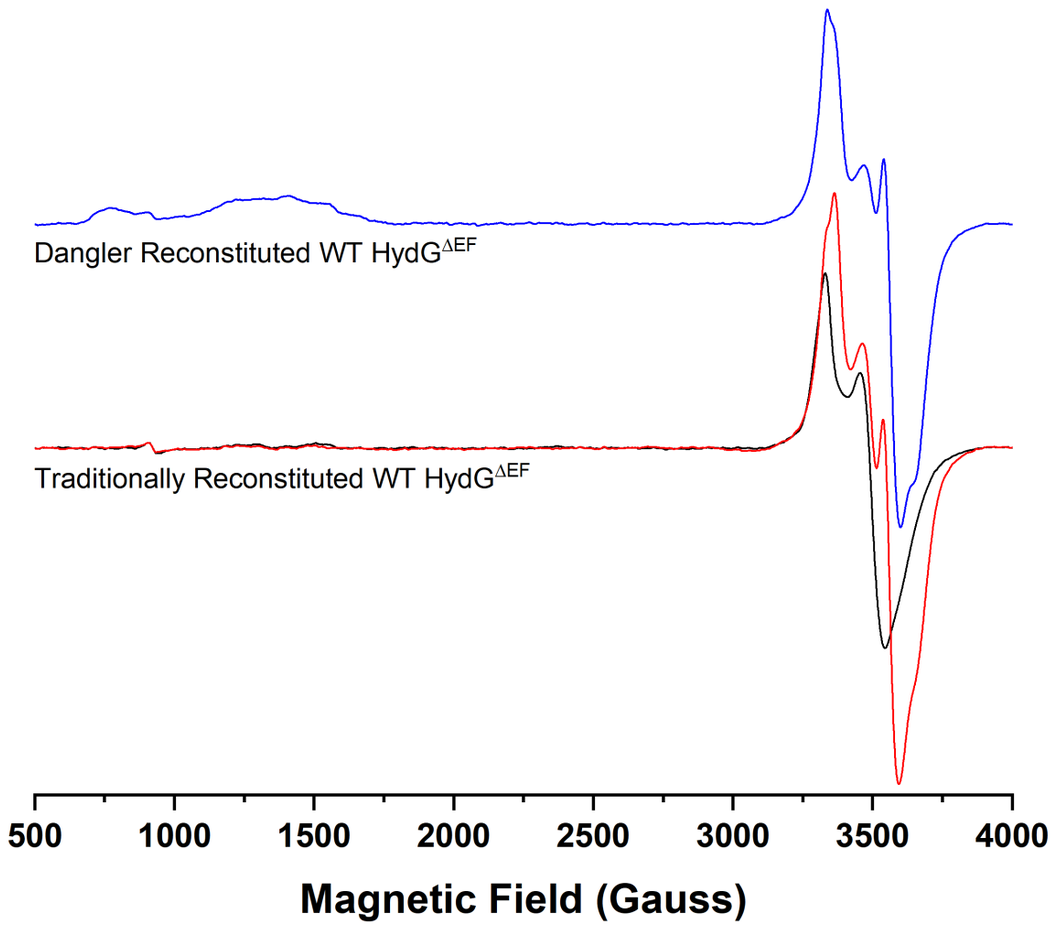

Figure S7. Wide field X-band, CW scans of HydG. Data are shown for traditionally reconstituted HydG (68 μM protein with 7.54 ± 0.48 Fe/protein) either in the presence of 3 mM DT (black) or 3 mM DT and 2 mM SAM (red). Also depicted is the data for the dangler reconstituted HydG (65 μM protein with 8.23 ± 0.43 Fe/protein) in the presence of 3 mM DT and 2 mM SAM (blue). All spectra were recorded at 10 G modulation amplitude, 10 K, 5.3 mW microwave power, and are the average of 3 scans.

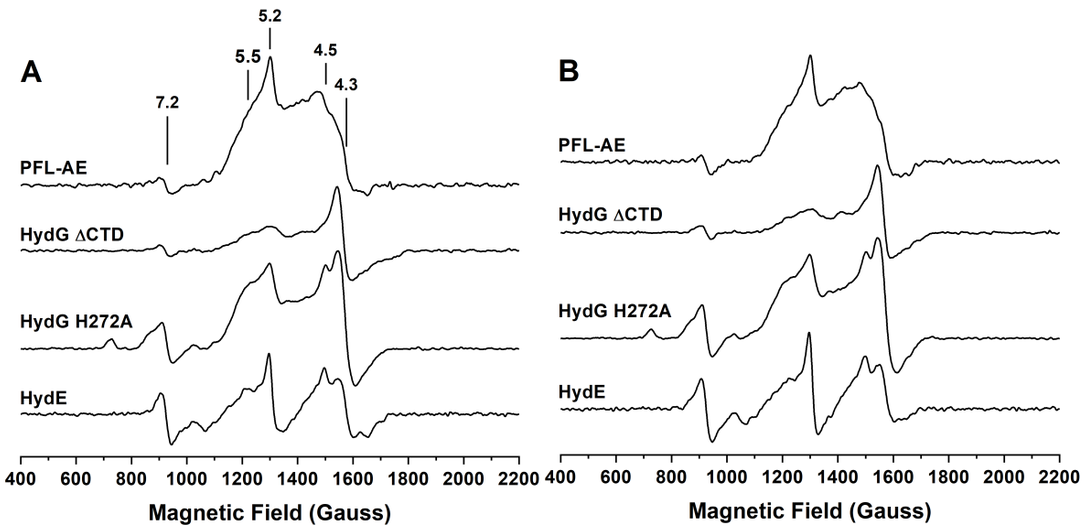

Figure S8. Low temperature CW X-band EPR spectra of HydG variant proteins, HydE, and PFL-AE. **A**. Spectra recorded at 8.0 K and 5.3 mW. **B**. Spectra recorded at 10.0 K and 5.3 mW. In both panels, spectra correlate to: traditionally reconstituted WT HydE (70 μM protein with 7.50 ± 0.20 Fe/protein, reduced with 3 mM DT in 50 mM Tris, 250 mM KCl, 5% glycerol, pH 8.0); traditionally reconstituted $HydG^{H272A}$ (100 μM protein with 7.65 ± 0.26 Fe/protein in 50 mM Tris, 10 mM KCl, pH 8.0 and reduced with 3 mM DT); traditionally reconstituted $HydG^{\Delta CTD}$ (210 μM protein with 2.40 ± 0.17 Fe/protein in 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol and reduced via photoillumination in the presence of 100 μM deazariboflavin and 5 mM DTT); and WT, as-purified PFL-AE (146 μM protein with 4.07 ± 0.43 Fe/protein in 50 mM Tris, 100 mM KCl, pH 7.5 and reduced via photoillumination in the presence of 50 μM deazariboflavin and 5 mM DTT).

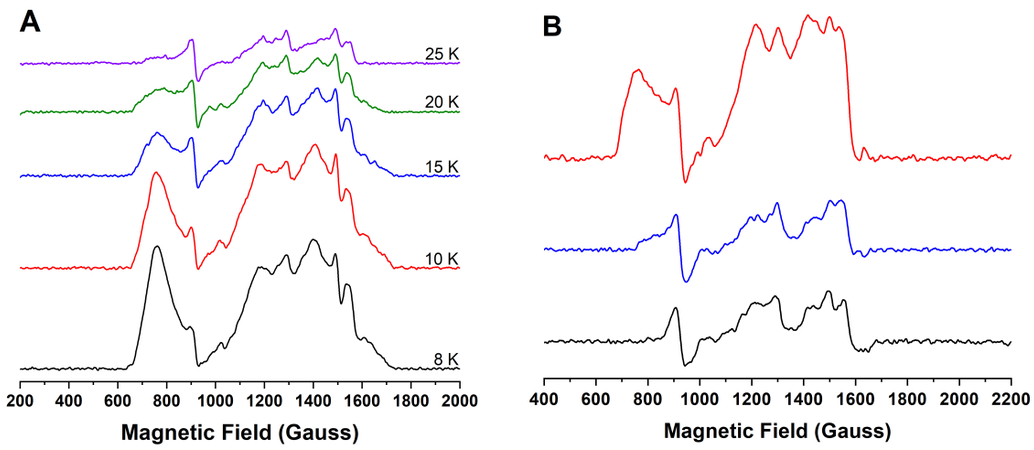

Figure S9. Low temperature, CW X-band EPR spectra of HydG enzyme preparations. **A**. WT dangler reconstituted HydG$^{\Delta EF}$ (55 μM protein with 8.79 ± 0.58 Fe/protein) reduced with 3 mM DT. Spectra were recorded at 5.3 mW microwave power at the temperature values noted in the panel. **B**. Spectra correlate to: WT traditionally reconstituted HydG$^{\Delta EF}$ (black, 74 μM protein with 7.54 ± 0.48 Fe/protein); WT traditionally reconstituted HydG^{EF} (blue, 64 μM protein with 7.38 ± 0.40 Fe/protein); and WT dangler reconstituted HydG$^{\Delta EF}$ (red, 60 μM protein with 8.23 ± 0.43 Fe/protein). Spectra were recorded at 10.0 K and 5.3 mW microwave power.

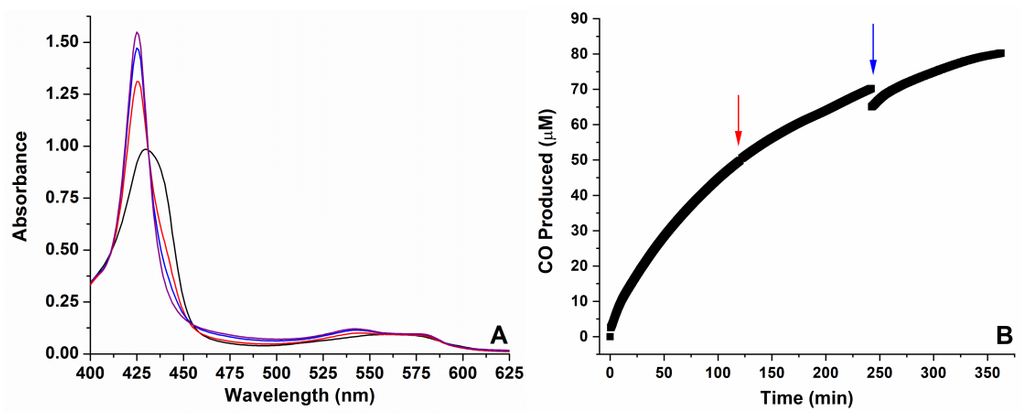

Figure S10. Exploring maximal amounts of exogenous CO that WT dangler loaded HydG is capable of generating during an in vitro assay. **A**. The HydG dependent H64L myoglobin Soret band shift over time for an assay performed in 50 mM Tris, 10 mM KCl, pH 8.1 buffer that contained WT HydG$^{\Delta EF}$ (10 μM protein with 8.23 ± 0.43 Fe/protein), 1 mM L-tyrosine, 1 mM SAM, 8 mM NaDT, and 86 μM H64L deoxyMb (per heme). Spectra correlate to: black, initial time = 0 min spectrum of HydG, dithionite, tyrosine, and H64L myoglobin; red, 120 minutes post addition of SAM; blue, 240 minutes post addition of SAM; purple, 362 minutes post addition of SAM). **B**. Single wavelength kinetics of CO formation as monitored via changes to the H64L myoglobin Soret band at 425 nm. The spectra in **A** were recorded at defined time points during the single wavelength kinetics experiment. The dangler reconstituted HydG$^{\Delta EF}$ protein (10 μM in assay) produced 32.1 μM CO in 60 min, 49.8 μM CO in 120 min, 69.9 μM CO in 240 min, and 80.3 μM CO in 362 min. The blue arrow in **B** denotes where a fresh aliquot of DT was added, followed by mixing via inversion of the cuvette.

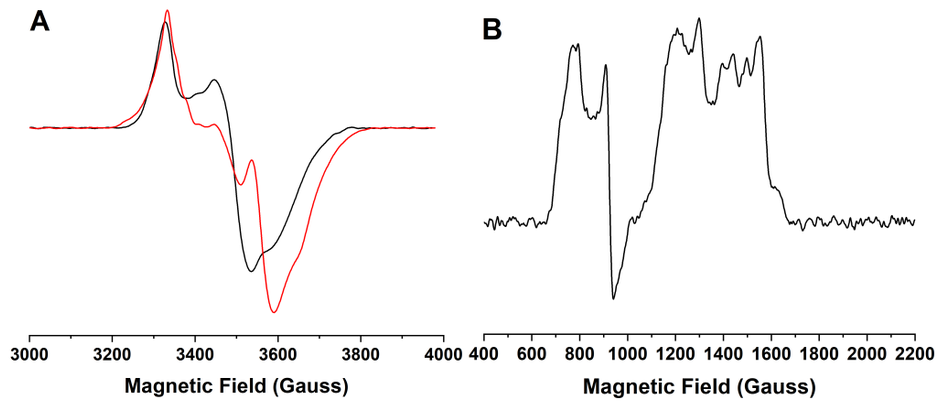

Figure S11. Low temperature CW X-band EPR spectra of WT strep-tagged HydG$^{\Delta EF}$. **A**. Spectra recorded at 10.0 K and 1.0 mW microwave power. **B**. Spectra recorded at 10.0 K and 5.3 mW microwave power. The black spectrum in both panels corresponds to the enzyme (58 μM protein with 9.20 ± 0.20 Fe/protein in 100 mM Tris pH 8, 250 mM KCl, 5% glycerol buffer) treated with 2 mM NaDT. In panel **A**, the red spectrum is the enzyme (58 μM protein with 9.20 ± 0.20 Fe/protein) treated with 2 mM NaDT and 1 mM SAM.

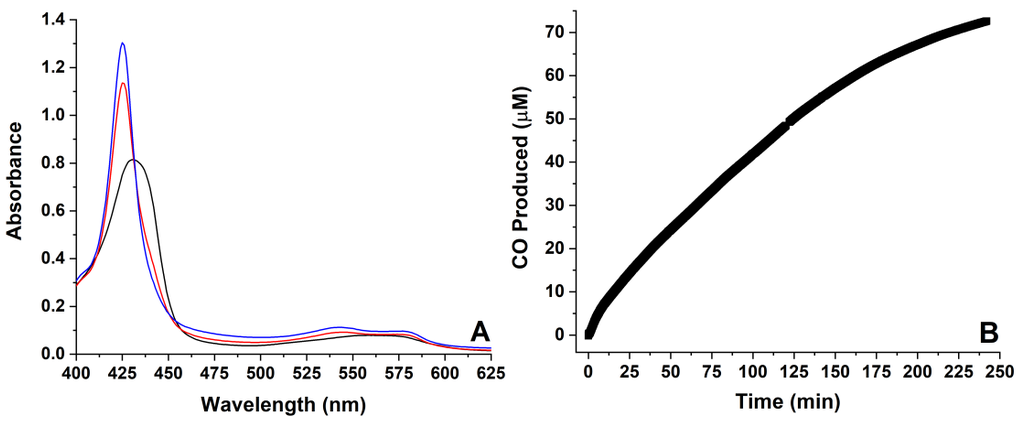

Figure S12. Strep-tagged HydG$^{\Delta EF}$ catalyzed CO formation kinetics, as monitored via binding to deoxy H64L myoglobin at 37 °C. **A**. H64L myoglobin (71 μM heme) in the presence of 8 mM NaDT, 600 μM L-tyrosine, and WT strep-tagged HydG$^{\Delta EF}$ (16.5 μM protein with 9.20 ± 0.20 Fe/protein, black spectrum). The red spectrum corresponds to the reaction mixture following addition of 1 mM SAM at the 2 hour time point, while the blue spectrum corresponds to the reaction mixture at the 4 hour time point post SAM injection. **B**. Single wavelength kinetics of CO formation as monitored via changes to the H64L myoglobin Soret band at 425 nm. The strep-tagged HydG$^{\Delta EF}$ protein produced 27.9 μM CO in 60 min, 48.5 μM CO in 120 min, and 72.6 μM CO in 240 min.

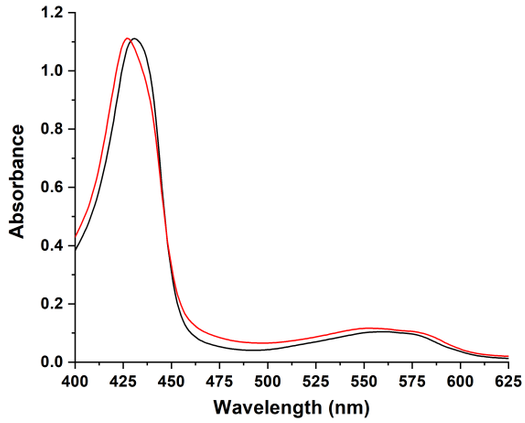

Figure S13. The HydG^{H272A} dependent H64L myoglobin Soret band shift over time. This figure depicts the results from an assay that was performed in 50 mM Tris, 10 mM KCl, pH 8.1 buffer and contained HydG^{H272A} (31 μM protein with 6.91 ± 0.14 Fe/protein), 1 mM L-tyrosine, 1 mM SAM, 5 mM NaDT, and 97 μM H64L deoxyMb (per heme). The spectra correlate to: black, initial time = 0 min spectrum of HydG^{H272A}, dithionite, tyrosine, and H64L myoglobin; red, 120 minutes post addition of SAM.

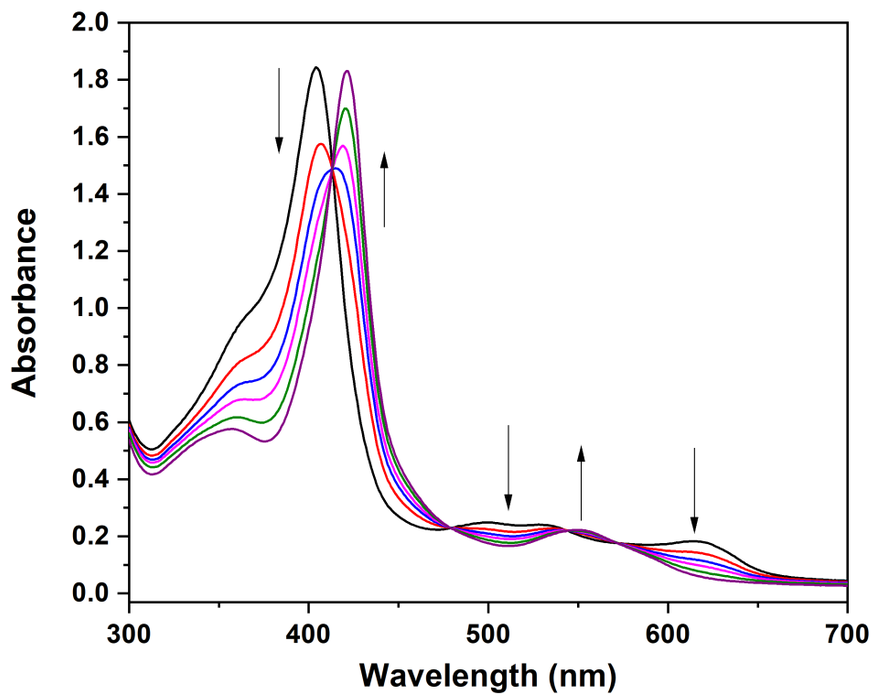

Figure S14. Addition of KCN (32 μM final) to purified P115A H-NOX protein (17 μM heme) in 50 mM HEPES, pH 7.4 buffer. The mixture was incubated at 60 °C for 4 hrs and the spectral changes over time were monitored. The initial spectrum prior to KCN addition is shown in black and is indicative of the Fe(III)-H_2O form of the enzyme with λ_{max}Soret = 404 nm and $\lambda_{max}\beta,\alpha$ = 528 nm and 614 nm, respectively. Following addition of KCN, isosbestic conversion to the Fe(III)-CN^- complex was observed with λ_{max}Soret = 421 nm and $\lambda_{max}\beta$ = 549 nm. Colored spectra correspond to different time points post addition of KCN (red, 15 min; blue, 32 min; magenta, 50 min; green, 90 min; purple, 4 hrs). A spectrum was also recorded at 5 hrs post KCN addition but there was no change relative to the 4 hr spectrum (data not shown).

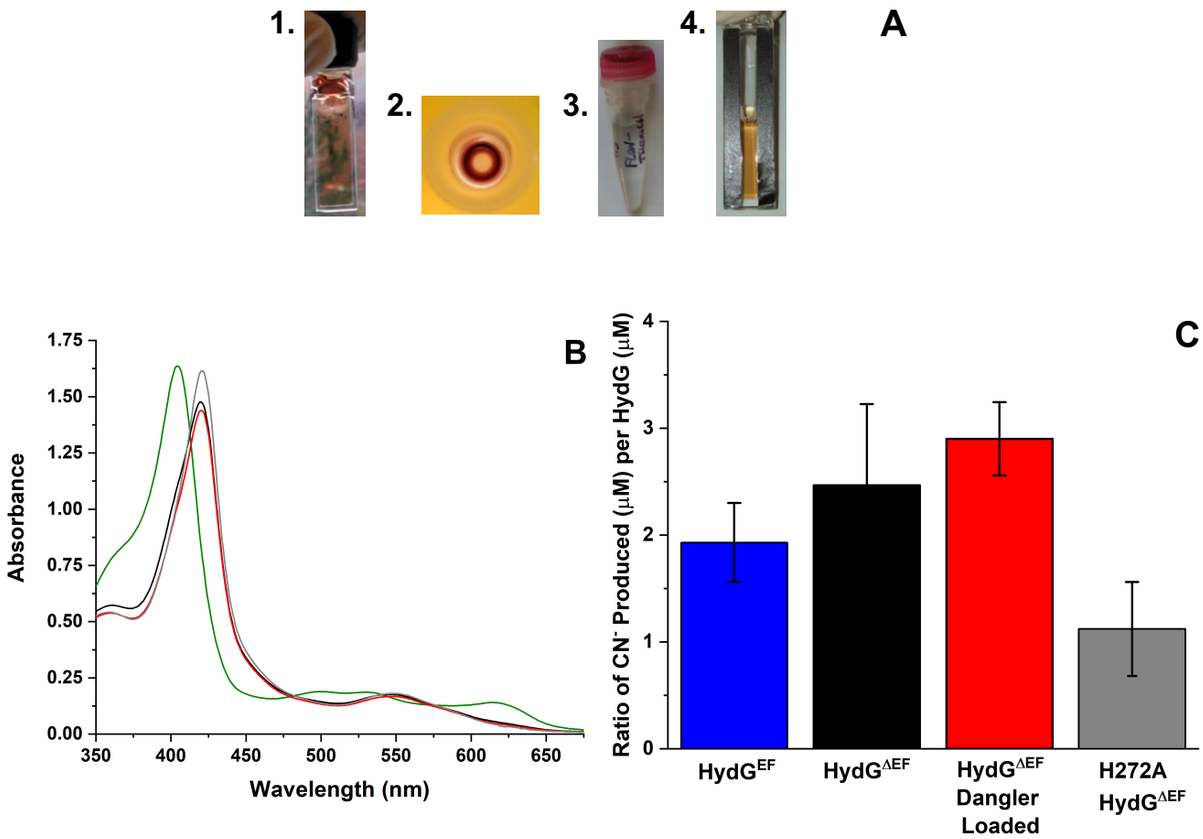

Figure S15. Spectroscopic analysis of HydG-MbCO assay filtrates for formation of free CN^-. **A**. Sample preparation. *1*. HydG-MbCO assay endpoint. *2*. An aliquot of the assay was first exposed to O_2 and then centrifuged under aerobic conditions using Nanosep 3,000 MWCO Omega™ (Pall) spin filters. *3*. The small molecular weight flow through component. *4*. The small molecular weight assay component was then added to a solution of Fe(III)-H_2O P115A H-NOX (15 μM heme) and was incubated at 60 °C for 4.5 hrs. The sample was visually observed to undergo the colorimetric change associated with Fe(III)-CN^- complex formation, which was subsequently confirmed via UV-VIS spectroscopic analysis. **B**. Spectroscopic changes associated with P115A H-NOX following the addition of HydG-MbCO assay filtrates; incubations performed in 50 mM HEPES, pH 7.4 buffer. The green spectrum is the control sample of the P115A H-NOX Fe(III)-H_2O form. The black spectrum is the resulting data from the coincubation of a MbCO–$HydG^{\Delta EF}$ (10 μM protein with 7.54 ± 0.48 Fe/protein) small molecular weight assay filtrate with Fe(III)-H_2O P115A H-NOX. The red spectrum is the resulting data from the coincubation of a MbCO–dangler loaded $HydG^{\Delta EF}$ (10 μM protein with 8.23 ± 0.43 Fe/protein) small molecular weight assay filtrate with Fe(III)-H_2O P115A H-NOX. The gray spectrum is the resulting data from the coincubation of a MbCO–H272A $HydG^{\Delta EF}$ (25 μM protein with 7.65 ± 0.26 Fe/protein) small molecular weight assay filtrate with Fe(III)-H_2O P115A H-NOX. Changes to the Soret band from the Fe(III)-H_2O P115A H-NOX to the Fe(III)-CN^- complexes (ΔAbs420 nm and Δε420 nm) were utilized to quantify the amount of Fe(III)-CN^- present. **C.** The CN^- quantities for the assays shown in the main body (4 hour assay time points, see Figure 5) are depicted as ratios over HydG enzyme.

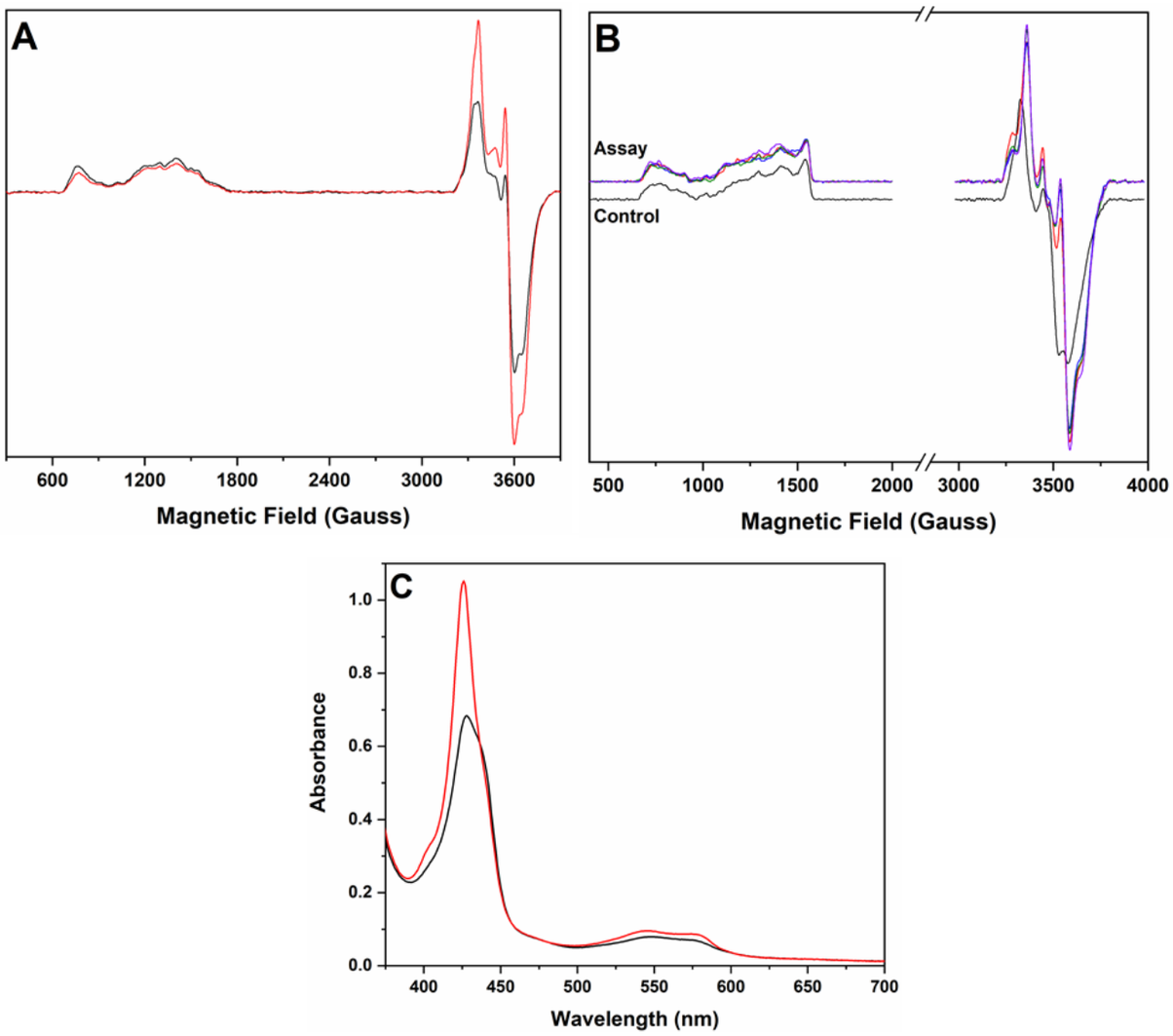

Figure S16. Spectroscopic characterization of HydG during turnover. **A**. Low temperature, CW X-band EPR spectra of the dangler loaded HydG that was utilized for the turnover experiment reported in **Figure 6**. HydG (86 μM, 9.08 ± 0.56 Fe/protein) is in the presence of 3 mM DT and 2 mM SAM. Wide field spectra were collected at a modulation amplitude of 10 G, modulation frequency of 100 kHz, and 5.3 mW microwave power at either 8 K (black) or 10 K (red); each spectrum is the average of 3 scans. **B**. EPR spectral changes during HydG turnover in the presence of H64L Mb. Dangler reconstituted HydG (22 μM protein with 9.08 ± 0.56 Fe/protein) was incubated in presence of 8 mM DT, 100 μM L-tyrosine, 1 mM SAM, and H64L Mb (60 μM heme). The control sample (black) lacked SAM. Colored spectra correspond to: red, 6 min; blue, 20 min; green, 60 min; purple, 120 min incubation periods at room temperature (see also **Figure 6**). The low field data were collected at 8 K and 5.3 mW microwave power (average of 6 scans), whereas the high field data were collected at 10 K and 1 mW microwave power (average of 6 scans). All spectra were collected at a modulation amplitude of 10 G, modulation frequency of 100 kHz, and were on the same gain setting, thus they are represented on the same intensity scale here. **C**. UV-Vis analysis of the control and assay (120 min) EPR samples in panel **B**. The EPR samples were thawed in the MBraun chamber, transferred to a 1 mM pathlength anaerobic cuvette, and the UV-Vis spectra were then recorded. The $\Delta A_{425\ nm}$ represents a 2.1-fold molar excess of CO generated in this experiment. The deoxy H64L Mb in the control sample clearly has a trace amount of the CO-bound form present. We propose that this arises from a small amount of SAM that remains associated with HydG following the dangler loading step (see Experimental), that was then consumed upon addition of L-tyrosine to this sample to generate a low amount of CO. The breadth of the control S=1/2 signals in the g = 1.84 region (**Figure 6B**) are consistent with this idea.