

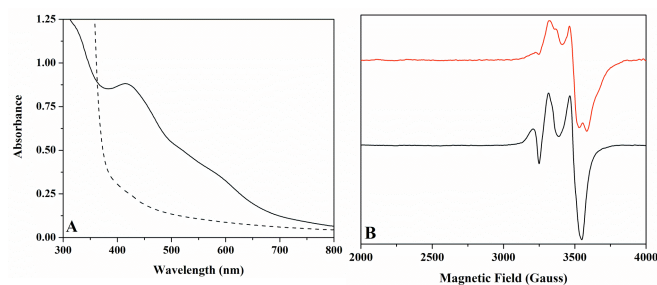
## SUPPORTING INFORMATION

### [FeFe]-Hydrogenase Maturation: HydG-Catalyzed Synthesis of CO

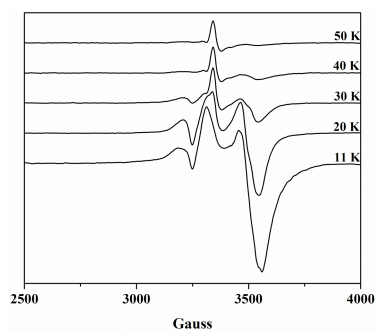
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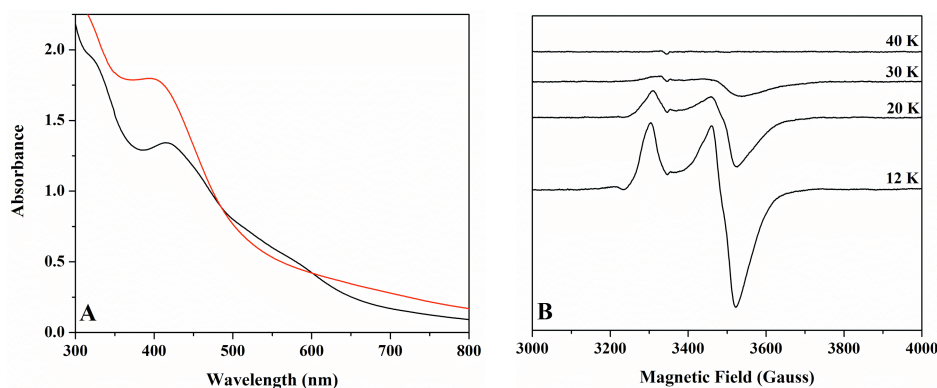
## SUPPLEMENTAL FIGURES



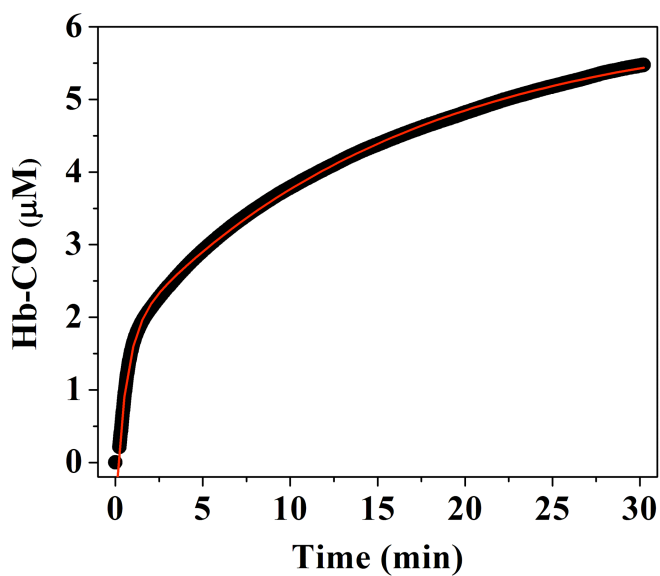
**Figure S1.** Spectroscopic characterization of non-reconstituted HydG. A. UV-visible spectra of HydG as-isolated (147  $\mu$ M,  $1.3 \pm 0.1$  Fe/protein, solid line) and after reduction using dithionite (44  $\mu$ M, 2.2 Fe/protein, dashed line). B. X-band EPR spectra of photoreduced HydG in the absence (black line, 284  $\mu$ M,  $2.2 \pm 0.3$  Fe/protein) and presence (red line, 279  $\mu$ M,  $2.2 \pm 0.3$  Fe/protein) of AdoMet. EPR parameters: 12 K, 9.36 GHz, 1.47 mW.



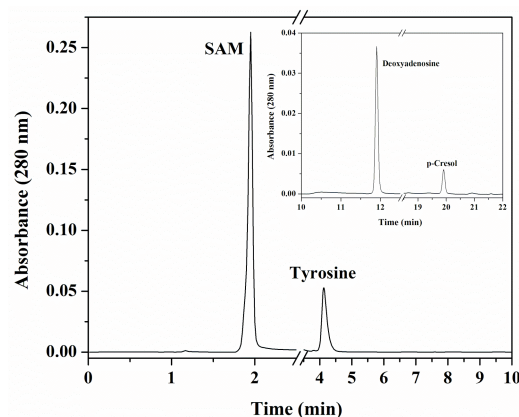
**Figure S2.** Temperature-dependence of the EPR signals present in non-reconstituted, photoreduced HydG. Sample shown at 11 K is the same sample shown in Figure S1, panel B, black line. EPR parameters: sample temp: 12 K, frequency: 9.36 GHz, power: 1.47 mW, time constant: 327.68 msec, conversion time: 81.92 sec.



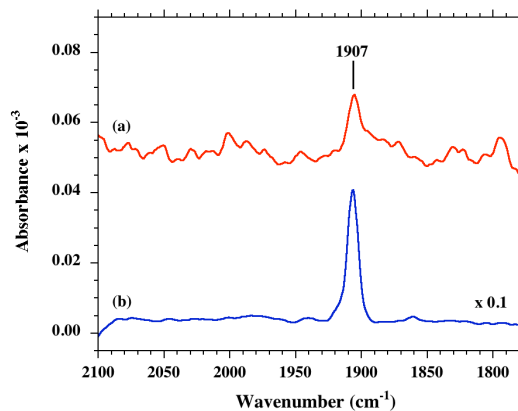
**Figure S3.** Spectroscopic characterization of reconstituted HydG. A. UV-visible spectra of non-reconstituted, as-isolated HydG (87  $\mu$ M protein,  $4.6 \pm 0.2$  Fe/protein, black line) and reconstituted HydG (63  $\mu$ M protein,  $6.8 \pm 0.1$  Fe/protein, red line). The  $\lambda_{\text{max}}$  shift from 415 nm to 395 nm was consistently observed in all samples following reconstitution. B. X-band EPR spectra of photoreduced, reconstituted HydG. The sample shown is the same as displayed in Figure 1 (65  $\mu$ M enzyme,  $8.7 \pm 0.7$  Fe/protein). EPR parameters are identical to those in Figure 1 and temperatures are indicated.



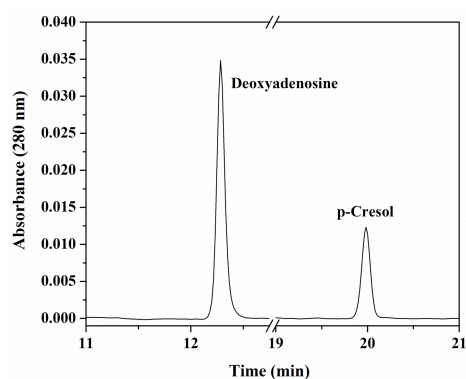
**Figure S4.** Single wavelength (419 nm) kinetics illustrating the biphasic nature of HbCO formation at 30°C. Reaction contained 33  $\mu\text{M}$  HydG ( $8.7 \pm 0.7$  Fe/protein), 4 mM dithionite, 1 mM Tyr, 1 mM SAM, 10.7  $\mu\text{M}$  heme. The fast phase results in 1.4  $\mu\text{M}$  HbCO in  $\sim 0.8$  min with HbCO formation continuing to 5.5  $\mu\text{M}$  in 30 min. The biexponential fit yielding the rates reported in the main text is shown in red (Origin 7.0).



**Figure S5.** HPLC analysis of HydG reaction products shown in Figure S4 (see SI text).



**Figure S6.** FTIR demonstrating the presence of  $^{13}\text{CO}$ -deoxyhemoglobin. (a) HydG reaction mixture containing U- $^{13}\text{C}$ -tyrosine, deoxyhemoglobin and SAM. (b) Control reaction mixture which contained all assay components as in (a) with the exception of U- $^{13}\text{C}$ -tyrosine. This control showed no active IR bands but was subsequently shot against 1 mM  $^{13}\text{CO}$  to yield spectrum b.



**Figure S7.** HPLC analysis demonstrating stoichiometric production of deoxyadenosine and p-cresol in a HydG CO assay (see SI text).

## SUPPLEMENTAL METHODS

### Protein Expression and Purification

Constructs encoding HydG from *Clostridium acetobutylicum* were transformed into *E. coli*- BL21(DE3) (Stratagene) cells for protein expression, as described previously with slight modifications.<sup>1</sup> Briefly, single colonies obtained from transformations were grown overnight in LB media and utilized to inoculate 9 L LB cultures containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L KCl, 5 g/L glucose and 50 mM potassium phosphate buffer pH 7.20. The cultures were grown at 37 °C and 225 rpm shaking until an OD<sub>600</sub>= 0.5 was reached at which point 0.06 g/L ferrous ammonium sulfate (FAS) and IPTG (1 mM final concentration) were added. The cultures were grown an additional 2.5 hours at 37°C, at which time an additional aliquot of 0.06 g/L FAS was added. The cultures were then transferred to a 10°C refrigerator and purged with N<sub>2</sub> overnight. Cells were harvested by centrifugation and the resulting cell pellets were stored at – 80°C until further use.

Cell lysis and protein purification were carried out under anaerobic conditions in a Coy chamber, as described<sup>1</sup> with slight modifications. Cell pellets were thawed and resuspended in a lysis buffer containing 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol, 10 mM imidazole, 20 mM MgCl<sub>2</sub>, 1 mM PMSF, 1% Triton X-100, 0.07 mg DNase and RNase per gram cell, ~ 0.6 mg lysozyme per gram cell. The lysis mixture was stirred for one hour, after which time the lysate was centrifuged in gas tight bottles at 18000 rpm for 30 minutes. The resulting supernatant was loaded onto a 5 mL HisTrapTM Ni<sup>2+</sup>-affinity column (GE Healthcare, Uppsala). The column was pre-equilibrated with 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol, 10 mM imidazole (buffer A). The column was subsequently washed with 15 column volumes of buffer A. Protein elution was accomplished by increasing the imidazole concentration in a stepwise manner from 10% to 20% to 50% to 100% buffer B (50 mM HEPES, pH 7.4, 0.5 M KCl, 5% glycerol, 500 mM imidazole). Fractions of interest were pooled, dialyzed into 50 mM HEPES, pH 7.4, 0.5 M KCl, 5% glycerol, and concentrated using an Amicon concentrator (Millipore; Bedford, MA) fitted with a YM-10 membrane or using a Prochem BJP 10/40 static protein concentrator. Protein was flash frozen in liquid N<sub>2</sub> and stored at – 80 °C or in liquid N<sub>2</sub> until further use.

### **Preparation of Reconstituted HydG**

Reconstitution of as-isolated HydG was carried out following the general procedures utilized for biotin synthase.<sup>2</sup> Enzyme (50 – 150 μM) was incubated with 6 – 7 fold excess of FeCl<sub>3</sub> and Na<sub>2</sub>S in the presence of 5 mM dithiothreitol (DTT) in 50 mM HEPES, 0.5 M KCl, 5% glycerol for ≈ 3 hours with gentle agitation in a Coy anaerobic chamber. Following a 5 minute

incubation with DTT,  $\text{FeCl}_3$  was added slowly and stirred for 20 minutes prior to the stepwise addition of  $\text{Na}_2\text{S}$ . Following the reconstitution period, the mixture was centrifuged and the supernatant was run over a Sephadex G-25 column (GE Healthcare) to remove excess ions. The resulting protein was concentrated using an Amicon concentrator fitted with a YM-10 membrane or a Pro-chem (Littleton, MA) BJP 10/40 static protein concentrator. UV-vis spectroscopy was utilized to confirm the [Fe-S] cluster content for reconstituted protein between the as-purified and as-reconstituted samples. Iron content of both the as-purified and reconstituted sample was examined by the spectrophotometric method developed by Fish, which uses ferrozine under reductive conditions after protein digestion with 4.5% (w/v)  $\text{KMnO}_4$  and 1.2 N  $\text{HCl}$ .<sup>3</sup>

UV-visible analysis of reconstituted HydG samples are brown in color and showed strong ligand-to-metal charge transfer bands at 320 and 415 nm that were more intense than the as-purified samples. Reconstituted samples used in this study had initial iron numbers between 2 to 3 per protein. Following reconstitution, iron analysis shows reconstituted samples to have iron numbers ranging between  $4.8 \pm 0.2$  to  $8.7 \pm 0.7$ .

### **UV-Visible Spectroscopic Analysis**

For UV-visible absorption experiments, samples were transferred to a 1.4 ml anaerobic cuvette (Spectrocell Inc, Orelan, PA) within an MBraun glove box (MBraun USA, Stratham, NH). Room temperature UV/vis absorption data were acquired using a Cary 6000i UV/vis/near-IR spectrophotometer (Varian, Palo Alto, CA). UV/vis spectra were collected at a data interval of 1.0 nm.



## **EPR Sample Preparation and Spectroscopic Analysis**

EPR samples were prepared in an MBraun box at O<sub>2</sub> levels <1 ppm. Photoreduced samples of non-reconstituted HydG (284  $\mu$ M; 2.22 Fe/protein) were made by supplementing the protein with 50 mM Tris, pH 7.4, 100  $\mu$ M deazariboflavin, and 5 mM DTT. Samples were then placed in an ice water bath in the MBraun box and illuminated with a 300 Watt Xe lamp for 1 hr. To determine the effects of SAM on reduced enzyme, an additional aliquot of protein was prepared simultaneously as described above. Following 1 hour photoreduction, 630  $\mu$ M SAM was added to the enzyme sample (279  $\mu$ M final; 2.22 Fe/protein) in the absence of light and the sample was immediately flash-frozen in liquid N<sub>2</sub>. Reconstituted samples of HydG (65  $\mu$ M protein at 8.7 Fe/protein) were prepared in an identical manner as described above, although the final concentration of SAM was 1 mM. Samples were stored in liquid N<sub>2</sub> until low temperature spectral analysis was performed (Figures 1, S1, S2 and S3). Low temperature EPR spectra were collected using a Bruker (Billerica, MA) EMX X-band spectrometer equipped with a liquid helium cryostat and temperature controller from Oxford instruments. Typical EPR parameters were: sample temperature, 12 K; microwave frequency, 9.37 GHz; microwave power, 1.84 mW; time constant, 81.92 msec, sweep time: 167.77 sec. Simulation of EPR data was performed with the EasySpin software program<sup>4</sup> and yielded the g-values reported in the main body.

## **CO Assays Additional Information**

CO formation assays were carried out in 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol. Raw data were corrected for the contribution of reduced HydG to the visible absorption. The  $\Delta A_{419}$  and  $\Delta \epsilon_{419}$ <sup>5</sup> were used to calculate the concentration of Hb-CO at each time point in order to determine the rate of CO formation. Data was fit to a biphasic exponential,

and  $k_{\text{cat}}$  values were calculated as previously described.<sup>6</sup> All kinetics assays were performed at 30 °C.

Control experiments lacking AdoMet were carried out using 36  $\mu\text{M}$  HydG at  $7.57 \pm 0.26$  Fe/protein in 3.22 mM DT and 10.5  $\mu\text{M}$  deoxyhemoglobin all in 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol buffer. Tyrosine (1 mM) was added and spectra were monitored for 2 hrs at 30 °C. No spectral changes were observed. At the 2 hr mark, the sample was transferred back to the MBraun box and 1.3 mM SAM was added. Within 15 minutes spectral changes associated with the Soret band consistent with HbCO formation were observed.

Control experiments lacking tyrosine were carried out using 36  $\mu\text{M}$  HydG at  $7.57 \pm 0.26$  Fe/protein in 3.22 mM DT and 10.5  $\mu\text{M}$  deoxyhemoglobin all in 50 mM HEPES pH 7.4, 0.5 M KCl, 5% glycerol buffer. AdoMet (1.3 mM) was added and spectra were monitored for 2 hrs at 30 °C. No spectral changes were observed. At the 2 hr mark, the sample was transferred back to the MBraun box and 1 mM tyrosine was added. Within 15 minutes spectral changes associated with the Soret band consistent with HbCO formation were observed.

Several attempts were made to simultaneously detect CO and  $\text{CN}^-$  in a single assay, however these were unsuccessful. One problem was buffer incompatibility, as the  $\text{CN}^-$  derivatization could not be carried out in Hepes buffer (the buffer used for CO assays), and the CO assays did not work in phosphate buffer (the buffer used for cyanide assays). Further, although the cyanide assay required protein denaturation and thus recovered and detected most of the cyanide produced, our method of utilizing hemoglobin to detect CO, together with the problems associated with quantifying a gaseous product, have prevented us from using a parallel denaturation protocol to detect CO. Efforts are currently underway to modify the CO assay in order to improve the quantitative aspects of the assay.

## FTIR Sample Preparation and Spectroscopic Analysis

Samples for FTIR analysis were prepared in an MBraun box at O<sub>2</sub> levels <1 ppm in a similar manner as for UV-vis analysis. Briefly, reconstituted HydG (59  $\mu$ M final concentration,  $8.1 \pm 1.7$  Fe/protein) was mixed with deoxyhemoglobin (115  $\mu$ M heme final) and U-<sup>13</sup>C-tyrosine (1 mM final) in 50 mM HEPES, pH 7.4, 0.5 M KCl, 5% glycerol, 4 mM sodium dithionite containing buffer. L-tyrosine (U-<sup>13</sup>C<sub>9</sub>, 97-99%; <sup>15</sup>N, 97-99%) at a chemical purity  $\geq 98\%$  was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). The mixture was set up in 1.0 mL screwcap cryovials and was incubated at 30 °C for 5 minutes using an IsoTemp block (Fisher, Waltham, MA). The assay was initiated by addition of AdoMet (1 mM final concentration) and following AdoMet addition the cryovial was immediately sealed. The assay was allowed to incubate for 1 hour at 30 °C at which time the mixture was flash frozen in liquid N<sub>2</sub>. Control mixtures were set up alongside the HydG-hemoglobin assay that utilized HydG from the same reconstitution batch but lacked either U-<sup>13</sup>C-labeled tyrosine or deoxyhemoglobin.

FTIR spectra were measured using a Bruker IFS/66S FTIR spectrometer interfaced to a home-built stopped-flow drive system with the sample cuvette and drive system maintained inside an anaerobic chamber (Belle Technology (Weymouth, UK), O<sub>2</sub> < 1.1 ppm) as described elsewhere.<sup>7</sup> The IR cuvette was thermostatted at 25°C. For these measurements, protein sample in 50 mM HEPES pH 7.4 buffer containing 500 mM KCl were introduced on only one side of the drive system, with the other side loaded with buffer. Spectra were measured at 4 cm<sup>-1</sup> resolution. The IR cuvette path length was calibrated at 47.6  $\mu$ m. Arbitrary background corrections were applied to yield flat baselines for measured spectra.

The presence of the vibrational band at  $1907\text{ cm}^{-1}$  in spectrum “a” is consistent with the existence of  $^{13}\text{CO}$  bound to the heme iron of deoxyhemoglobin (Figure 2A inset and Figure S6). Neither control showed the presence of a band at  $1907\text{ cm}^{-1}$ , and differential spectral analysis yielded the spectra in Figure S6. As an internal control experiment, exogenous  $^{13}\text{C}$ -labeled CO (1 mM) was shot against the control sample that lacked  $^{13}\text{C}$ -labeled tyrosine, resulting in spectrum “b” in Figure S6. Based on peak area relative to the control  $^{13}\text{CO}$  spectrum, we estimate  $\approx 4\text{ }\mu\text{M}$  HbCO formation in spectrum “a”.

### **HPLC Analysis of HydG Reaction Products**

At given time points, aliquots of the HydG, deoxyhemoglobin assay mixtures were removed and quenched by addition of 1 M HCl. Samples were then boiled for 40 seconds, and centrifuged two subsequent times at 14000 rpm at  $4\text{ }^{\circ}\text{C}$  and the supernatant was subjected to HPLC analysis. Initial sample aliquots were run through a YM-3 Microcon filter (Millipore) prior to subjecting the flow through to HPLC analysis. All subsequent samples were not subjected to YM-3 filter treatment, as it was discovered that p-cresol binds to the YM-3 filter (see below). Samples were injected onto a Phenomenex (Torrance, CA) Curosil  $5\text{ }\mu\text{m}$   $4.6 \times 150$  mm analytical column that had been equilibrated at 98% solution A ( $\text{H}_2\text{O}$  plus 0.1% acetic acid) and 2% solution B ( $\text{CH}_3\text{CN}$  plus 0.1% acetic acid) at 1 ml/min. An isocratic mobile phase at these percentage values ran for 7 minutes following sample injection at which time a linear gradient to 40% solution A, 60% solution B was run over the next 17 minutes. At this time the gradient was held isocratic for 3 minutes and then the column was re-equilibrated to 98% solution A and 2% solution B for another sample injection. The column temperature during runs was held at  $30\text{ }^{\circ}\text{C}$  and total run time was 35 minutes. Elution of reaction products was monitored

via absorbance at 280 nm and under these conditions AdoMet eluted at  $\sim 2$  minutes, tyrosine eluted at  $\sim 4$  minutes, deoxyadenosine eluted at  $\sim 12$  minutes, and p-cresol eluted at  $\sim 20$  minutes (Figure S5). Integration of peak area using Origin 7.0 (Microcal, MA, USA) relative to standard samples run in parallel allowed for quantification of reaction product concentrations.

Figure S5 shows the reaction components from the experiment shown in Figure S4, including unreacted AdoMet and tyrosine and the reaction products deoxyadenosine and p-cresol. Absorbance changes for the assay mixture in Figure S4 were monitored for a total time of 1.5 hours yielding a final HbCO concentration of  $6.7 \mu\text{M}$  (data not shown). HPLC injections were performed in triplicate and quantification of deoxyadenosine produced during this time was shown to be  $103.6 \pm 6.6 \mu\text{M}$ , while p-cresol formed was found to be  $55.1 \pm 5.5 \mu\text{M}$ . Sample aliquots for this analysis had been subjected to treatment with a YM-3 filter membrane and following HPLC analysis, it was demonstrated using control solutions that approximately 50% of an applied concentration of p-cresol would bind to the YM-3 filter membrane. All subsequent aliquots for HPLC analysis, such as those shown in Figures 2C and S7, were not subjected to YM-3 filter treatment. Adjusting the yield of p-cresol for this fact shows that the  $33 \mu\text{M}$  enzyme in Figure S5 underwent a total of  $\sim 3.08$  turnover events during 1.5 hrs, which agrees quite well with levels of turnover reported by the Roach laboratory.<sup>8</sup>

Figure S7 shows the end result of a similar experiment. In this case,  $64 \mu\text{M}$  HydG ( $6.4 \pm 0.2 \text{ Fe/protein}$ ) produced  $9.5 \mu\text{M}$  HbCO in 75 minutes. The amounts of deoxyadenosine and p-cresol were determined to be  $117.5 \pm 10.8 \mu\text{M}$  and  $102.1 \pm 5.9 \mu\text{M}$ , respectively. Although lower total turnover was observed in this experiment, which is likely due to the lower iron number of the enzyme sample, it demonstrates nearly stoichiometric deoxyadenosine:p-cresol formation.

Given the consistent results that showed substoichiometric HbCO formation relative to levels of deoxyadenosine and p-cresol, we designed an experiment to determine levels of HydG reaction products formed within 5 minutes. Figure 2B and 2C shows the results of an experiment where 45  $\mu\text{M}$  HydG ( $7.2 \text{ Fe/protein} \pm 0.21$ ) was incubated with 10  $\mu\text{M}$  heme deoxyHb, 1 mM AdoMet and 500  $\mu\text{M}$  tyrosine. The experiment was set up to run in single wavelength absorbance mode (at 419 nm) using a Cary spectrophotometer (similar to the data shown in Figure S4). At 1 minute time the program was paused and an aliquot of the reaction mixture was removed using a Hamilton (Reno, NV) gas tight syringe (denoted by an arrow in panel B). This aliquot was immediately quenched with 1 M HCl and flash frozen in liquid  $\text{N}_2$ . The program was reinitiated and at 2 minutes and 5 minutes additional aliquots were taken as described. These aliquots were prepared for HPLC analysis as described above, avoiding the use of YM-3 filter membranes (Figure 2C).

The data clearly shows that the amount of HbCO formed is less than the amount of deoxyadenosine and p-cresol produced. At 1 minute, we observe  $\sim 1 \mu\text{M}$  HbCO to  $7.3 \pm 0.33 \mu\text{M}$  p-cresol and  $8.9 \pm 0.04 \mu\text{M}$  deoxyadenosine. At 2 minutes, we observe  $1.4 \mu\text{M}$  HbCO,  $11.9 \pm 1.90 \mu\text{M}$  p-cresol,  $11.2 \pm 0.15 \mu\text{M}$  deoxyadenosine and at 5 minutes we see  $2.2 \mu\text{M}$  HbCO,  $16.9 \pm 0.17 \mu\text{M}$  p-cresol, and  $19.5 \pm 0.38 \mu\text{M}$  deoxyadenosine formation. We hypothesize that the low levels of CO relative to deoxyadenosine and p-cresol may represent sequestration of some CO within HydG itself. The assays developed by the Roach laboratory for cyanide detection used acid denaturation of HydG and may therefore explain the greater levels of cyanide detection, especially in light of the similar levels of deoxyadenosine and p-cresol observed in our enzyme preparations that yield CO.

## SI References

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