Supporting Information

Reversible H Atom Abstraction Catalyzed by the Radical SAM Enzyme HydG

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Figure S1. ESI-MS of HydG product 5'-dAdoH isotope distribution from assays performed in H2O buffer (50 mM tris, 300 mM KCl, 5% glycerol, pH 8.1) with tyrosine isotopologs. (A) [β-D2]-Tyr (B) [ring-D4]-Tyr (C) [α,ring-D5]-Tyr (D) Unlabeled Tyr. Samples were incubated for 37 °C for 60 minutes in mixtures containing HydG (100 µM; 4.4 ± 0.3 Fe/protein), 1 mM Tyr, 1 mM SAM, and 5 mM dithionite, as was described in the main text. Spectra are scaled, extracted ion chromatograms.
Figure S2. GC-MS of HydG product p-cresol from assays performed in H2O buffer (50 mM tris, pH 8.1) with tyrosine isotopologs. (A) [ring-$D_4$]-Tyr (B) [α,ring-$D_5$]-Tyr (C) [β-$D_2$]-Tyr (D) Natural abundance p-cresol standard in H2O buffer. Samples were incubated for 37 °C for 60 minutes in mixtures containing HydG (100 µM; 4.4 ± 0.3 Fe/protein), 1 mM Tyr, 1 mM SAM, and 5 mM dithionite, as was described in the main text. Spectra represent extracted ion chromatograms.
Figure S3. Control ESI-MS of SAM isotope distributions performed in 95% D₂O buffer (50 mM tris, pH 8.1) under assay conditions but lacking essential reaction components. (A) Sample lacking Tyr. (B) Sample lacking HydG. (C) SAM Reference in H₂O. Samples were incubated for 37 °C for 60 minutes to contain (except as noted) HydG (100 µM; 8.5 ± 0.1 Fe/protein), 1 mM Tyr, 1 mM SAM, and 5 mM dithionite, as was described in the main text. Spectra are represented as normalized, extracted ion chromatograms.
Figure S4. GC-MS of HydG product p-cresol under assay conditions containing all reaction components in 95% D$_2$O buffer (50 mM tris, pD 8.1). (A) HydG Assay Sample. (B) p-cresol standard prepared in 95% D$_2$O buffer (50 mM tris, pD 8.1). (C) p-cresol standard prepared in H$_2$O buffer. The assay sample (A) contained HydG (100 µM; 8.5 ± 0.1 Fe/protein), 1 mM Tyr, 1 mM SAM, and 5 mM dithionite, as described in the main text, and was incubated at 37 °C for 60 minutes. All sample volumes were quenched or diluted 1:1 with neat acetonitrile. Spectra are represented as extracted ion chromatograms.
Supporting Tables

Table S1. Isotope Distribution of 5’-deoxyadenosine (dAdoH) (as percent of total)\(^a\)

| Sample  | 5’-dAdo–CH\(_3\) | 5’-dAdo–CH\(_2\)D | 5’-dAdo–CHD\(_2\) | 5’-dAdo–CD\(_3\) |
|---------|------------------|-------------------|-------------------|------------------|
| Figure 3A | 18               | 33               | 27                | 22               |

\(^a\) No dAdoH was detected when either Tyr or HydG was omitted from the reaction under these conditions.

Table S2. Percent Sample Isotope Distribution of S-adenosylmethionine (SAM)

| Sample   | SAM–CH\(_2\) | SAM–CHD | SAM–CD\(_2\) |
|----------|--------------|---------|--------------|
| Figure 3A | 44           | 37      | 19           |
| Figure S3A | 90           | 10      | 0            |
| Figure S3B | 91           | 9       | 0            |
Supporting Methods

Materials:

All chemicals and other materials used herein were from commercial sources and of the highest purity where available. Natural abundance tyrosine was obtained from Sigma-Aldrich (St. Louis, MO). \([\beta-D_2]\)-tyrosine was obtained from Cambridge Isotope Laboratory (Tewksbury, MA). \([\text{ring-}D_4]\)-tyrosine and \([\alpha,\text{ring-}D_5]\)-tyrosine was obtained from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Tris, HEPES, and DTT were obtained from RPI (Mt. Prospect, IL). KCl, acetonitrile (HPLC grade) and glycerol were obtained from EMD (Gibbstown, NJ). Sodium dithionite, D_2O, and sodium sulfide was obtained from Acros Organics (Fair Lawn, NJ). Iron(III) chloride and acetic acid (99%, HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ). SAM\(^1\) was purified as reported elsewhere, with slight modifications.

HydG Overexpression, Purification, Reconstitution, and Assessment of Activity:

Heterologous overexpression of *Clostridium acetobutylicum* HydG in *Escherichia coli*, purification and chemical reconstitution with iron and sulfide were prepared as described previously with slight modifications.\(^2,3\) 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 7.2. The cultures were grown at 37 °C and 225 rpm shaking until an OD\(_{600}\) = 0.5 was reached at which point 0.06 g/L ferrous ammonium sulfate (FAS) and isopropyl \(\beta\)-D-1-thiogalactopyranoside (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\(_2\) 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 (Grass Lake, MI), as described with slight modifications. \(^2\) \(^3\) Cell pellets were thawed and resuspended in a lysis buffer containing 50 mM HEPES, 500 mM KCl, 5% glycerol, 10 mM imidazole, pH 7.4, 20 mM MgCl\(_2\), 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 18,000 rpm for 30 minutes. The resulting supernatant was loaded onto a 5 mL HisTrap\textsuperscript{TM} Ni\textsuperscript{2+}-affinity column (GE Healthcare, Uppsala). The column was pre-equilibrated with 50 mM HEPES, 500 mM KCl, 5% glycerol, 10 mM imidazole, pH 7.4 (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, 500 mM KCl, 5% glycerol, 500 mM imidazole, pH 7.4). Pure fractions (gauged by SDS-PAGE) were dialyzed into 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 and concentrated using an Amicon Ultra centrifugal unit (Millipore; Billerica, MA) fitted with a YM-10 membrane or using a Minicon B15 static protein concentrator (Millipore). Protein was flash frozen in liquid N\(_2\) and stored at –80 °C until further use.

Reconstitution of as-purified HydG was carried out following the general procedures described previously. \(^3\) Enzyme (50 – 150 µM) was incubated with 6 – 7 fold excess of FeCl\(_3\) and Na\(_2\)S in the presence of 5 mM dithiothreitol (DTT) in 50 mM HEPES, 500 mM KCl, 5% glycerol, pH 7.4 for ≈ 3 hours with gentle agitation in a Coy anaerobic chamber at 22 °C. Following a 5 minute incubation with DTT, FeCl\(_3\) was added slowly and stirred for 20 minutes...
prior to the stepwise addition of Na$_2$S. Following the reconstitution period, the mixture was centrifuged to remove exogenous FeS clusters in the mixture. Following initial concentration using an Amicon centrifugal unit, the supernatant was treated over a Sephadex G-25 column (GE Healthcare; Piscataway, NJ) to remove excess ions. Following this, the darkest brown fractions were pooled and concentrated using an Amicon centrifugal unit. UV-visible spectroscopy was utilized to confirm the Fe–S cluster content for reconstituted protein between the as-purified and as-reconstituted samples. Enzyme concentration was determined by performing a Bradford assay,\textsuperscript{4} while the iron content was determined through the colorimetric ferrozine method.\textsuperscript{5}

HydG activity was determined by performing by assessing CO production using methods described previously.\textsuperscript{3} Briefly, typical samples contained 40 $\mu$M HydG with 1 mM Tyr, 1 mM AdoMet, 80 $\mu$M deoxyhemoglobin, and 5 mM dithionite, in 50 mM tris, 300 mM KCl, pH 8.1 (400 $\mu$L total volume), and was prepared in an anaerobic chamber. CO experiments were performed using a Cary 600i dual pathlength UV-visible Spectrophotometer (Agilent; Santa Clara, CA) in a 1 mm pathlength anaerobic cuvette (Spectrocell; Oreland, PA) fitted with a teflon membrane. Upon performing an initial 300-800 nm scan (600 nm/min), SAM was added using a stoppered 25 $\mu$L gastight syringe (Hamilton; Reno, NV). Upon addition and mixing, 419 nm absorbance (the Soret $\lambda_{\text{max}}$ for carboxyhemoglobin) was monitored every second for 30 min at 37 °C.\textsuperscript{6} Following this, a final 300–800 nm scan was made. Enzyme stocks used for timecourse and H-atom abstraction experiments described herein produced on upwards of 30 $\mu$M CO after 30 min at 37 °C, under the conditions reported above.

**H Atom Abstraction Experiments:**

Assays were performed in either H$_2$O or D$_2$O buffer. Experiments were performed in an anaerobic chamber (Mbraun; Stratham, NH) under strict anaerobic conditions (< 1 ppm O$_2$). H$_2$O
buffers were prepared by degassing on a schlenk line (3 x 10 min vacuum cycles followed by N2 backfill). Buffer volume lost to evaporation was replaced with anaerobic H2O in the glovebox. D2O buffers were prepared by lyophilization of H2O buffer on a Schlenk line in a Schlenk flask for 16 hrs. The resultant salt was brought into the glovebox and was dissolved with degassed D2O to original volume. Prior to suspending the salt in D2O, the Schlenk flask was briefly subjected to a heat gun under vacuum to drive off remaining H2O moisture. Upon resuspending the buffer salt in D2O, the pD was measured with pH paper (Micro Essentials; Brooklyn, NY) and was as expected acidic by 0.4 units.7,8 Working 2 mM Tyr stocks in D2O were prepared in a similar fashion to buffers described above, via dilution from an aerobic 67.7 mM stock in H2O (prepared by dissolving Tyr in 1 M HCl, then dilution with 100 mM tris, pH 7.4) that was lyophilized overnight.

Experiments were performed at 37 °C in an IsoTemp heatblock (Fisher), containing 100 µM HydG (8.5 ± 0.2 Fe/protein), 1 mM AdoMet (enzymatically prepared), 1 mM Tyr, and 5 mM dithionite (80 µL volume) for 60 minutes, where the enzyme was precipitated via 1:1 volume addition of acetonitrile (HPLC grade, EMD) or addition of 1 M HCl (13 % v/v). A concentrated HydG stock at ~ 2 mM was used to minimize the H2O contribution for performed experiments. It should be noted that use of either quenching medium noted above did not affect the observed product isotope distribution. Samples were centrifuged for 3 x 10 minutes at 14,000 rpm, where the supernatant was collected. Samples were then used for LC-MS or HPLC, as described below. Reported assay pDs or pHs were corrected for changes related to temperature.9

**HydG Time Course Assays:**

Time-dependent production of 5′-dAdo and p-cresol by HydG was performed in a manner similar to H-atom abstraction experiments performed above, in an anaerobic chamber.
Assays were performed in duplicate at 37 °C in a heat block containing 40 µM HydG (9.5 ± 0.2 Fe/protein), with 1 mM AdoMet, 1 mM Tyr, 5 mM dithionite, in 50 mM tris, 300 mM KCl, pD/pH 8.1 buffer (80 µL total volume). Reported assay pDs or pHs were corrected for changes related to temperature. Experiments were initiated by the addition of AdoMet, and samples were incubated for 30 sec, 1 min, 2 min, 5 min, 10 min, 15 min, 30 min, or 60 minutes before quenching by the addition of 1 M HCl (13% v/v). Samples were centrifuged for 3 x 10 min to ensure removal of the precipitated enzyme. Samples were injected onto a Phenomenex (Torrance, CA) Kinetex PFP analytical column (150 x 4.60 mm) connected to an Agilent 1100 series HPLC containing an autosampler. The column was equilibrated with 98% solution A (H₂O + 0.1% acetic acid) and 2% solution B (CH₃CN + 0.1% acetic acid) at 1 mL/min. Following sample injection, the mobile phase was held isocratically for 7 minutes, where a linear gradient to 40% solution A, 60% solution B was run over the next 17 minutes. At this point, the gradient was held isocratically for 3 minutes before the column was re-equilibrated to 98% solution A and 2% solution B for subsequent sample injections. The column temperature was held at 30 °C and the total run time was 35 minutes. Product detection was monitored at 280 and 254 nm. AdoMet eluted at ~ 2 minutes, tyrosine eluted at ~ 4 minutes, deoxyadenosine eluted at ~ 12 minutes, and p-cresol eluted at ~ 18 minutes. Integration of peak area relative to standard samples ran in parallel using Agilent’s ChemStation data analysis package allowed for quantitation of reaction product concentrations, and data was fitted using OriginPro v. 8.6 (OriginLab Corp; Northampton, MA).

**LC-MS Methods:**

Deuterium isotope distribution in AdoMet and 5’-dAdo was assessed using LC-MS. After centrifugation of the precipitated HydG, samples prepared in H₂O were diluted 100-fold in
10% methanol; D$_2$O samples were treated without further dilution. Quantitation of 5’-deoxyadenosine, AdoMet, and tyrosine was performed using an Agilent 1290 series UHPLC coupled to an Agilent 6538 Q-TOF mass spectrometer equipped with the dual-ESI source and an autosampler. Samples were injected onto a normal phase Microsolv (Eatontown, NJ) Cogent “Diamond Hydride” HPLC column (150 x 2.1 mm) equilibrated with solution B (CH$_3$CN + 0.1% formic acid) at 0.8 mL/min. An isocratic mobile phase was maintained for 2 minutes following sample injection, and then a linear gradient from solution A (H$_2$O + 0.1% formic acid) to 50% solution B was run for 4 minutes. This was followed by isocratic elution for 2.5 minutes at 50% solution B after which it was immediately re-equilibrated to 100% B for 1.5 minutes. Total run time was 10 minutes and the column temperature was maintained at 50 °C. Under these conditions, 5’-dAdo eluted at ~ 3 minutes, AdoMet eluted at ~ 6 minutes, and tyrosine eluted at ~ 4 minutes. The capillary exit voltage was 120 V and gas temperature was 300 °C. All data was recorded in positive mode between 25 m/z and 750 m/z in profile mode. Hardware summation time was 1 second. Peaks were validated using high-accuracy formula confirmation and elution matching from individual reference standards. Quantitation was performed using the MassHunter Quantitative Analysis package (Agilent). An extracted ion chromatograph was generated using the accurate mass of each compound with a -0.04 to +0.04 ppm m/z extraction window. Samples were injected in duplicate, and between sample injections, a water blank injection was performed to minimize sample-to-sample carryover.

Sample isotope distributions for 5’-dAdo and SAM were assessed using the natural abundance distribution as a template distribution. Incorporation of a deuterium atom corresponds to a net mass increase of one, but results in an otherwise identical distribution to the
template distribution. Respective isotopic mass abundances were subtracted, and quantities for each were normalized to 100% total.

In addition to the experiments described in the main text, samples were also quenched immediately after AdoMet was added, to assess deuterium label incorporation. Negligible 5’-dAdo product was observed after HCl was added ~ 2 seconds after AdoMet was added, and the observed AdoMet isotope distribution was identical to a reference standard prepared in H2O. For experiments performed in D2O, no label transfer was observed on tyrosine substrate. The mass spectrum of Tyr was found to be identical to a reference standard prepared in H2O. Finally, to investigate non-enzymatic solvent exchange of SAM, a sample containing only SAM in D2O buffer was prepared and was immediately subjected to LC–MS. Because the column was equilibrated in protic solvent, the detected SAM was identical to the respective standard prepared in H2O buffer.

**GC-MS Methods:**

Isotope distributions of p-cresol product formed were determined using an Agilent 7890A GC system coupled to a 5975C inert XL EI/CI MSD with a triple axis detector. 1 µL of samples that were subjected to LC-MS described above were injected onto an Agilent HP-5ms column (30 m x 0.25 mm i.d.; 250 micron thickness) pre-equilibrated at 100 °C. The column temperature was maintained at 100 °C for 1 minute, before the temperature was increased by 15 °C per minute until reaching 300 °C. The temperature was maintained for 3 minutes, constituting the end of the run. p-Cresol had a retention time of 3.9 minutes. Ion extraction and data manipulation was performed using the Agilent MassHunter Qualitative Analysis package.
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