Supporting Information

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Abstract: Radical S-adenosyl-l-methionine (SAM) enzymes initiate biological radical reactions by using a $[4Fe\text{-}4S]^{+}$ cluster to reductively cleave SAM via the Ω organometallic intermediate, in which the 5'-deoxyadenosyl moiety is directly bound to the unique iron of the [4Fe-4S] cluster. Here we present synthesis of the SAM analog S-adenosyl-l-ethionine (SAE) and show SAE serves as a mechanistically equivalent SAM alternative for HydG, both supporting enzymatic turnover of substrate tyrosine and forming the organometallic intermediate Ω. Photolysis of SAE bound to HydG results in formation of an ethyl radical trapped in the active site. The ethyl radical withstands prolonged storage at 77 K and its EPR signal is only partially lost upon annealing at 100 K, making it significantly less reactive than the methyl radical reported previously. Upon annealing above 77K, the ethyl radical adds to the $[4Fe\text{-}4S]^{2+}$ cluster to generate an ethyl-$[4Fe\text{-}4S]^{3+}$ organometallic species termed Ω_E.

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Experimental Procedures

HydG was expressed, purified, and reconstituted as previously described.[1] All chemicals were obtained from commercial sources unless otherwise stated.

General Methods. ^{1}H NMR spectra were obtained on a Bruker AVANCE III HD 600 MHz NMR spectrometer. Chemical shifts are listed in parts per million and were reported relative to TMS by referencing the residual solvent. Coupling constants (*J*) are listed in Hz. Liquid chromatography- mass spectrometry (LC-MS) analysis of SAE was performed using an Agilent 6538 UHD Accurate-Mass QTOF LC/MS in positive mode.

Preparation of SAE. Synthesis of *S*-adenosylethionine (SAE) was accomplished following a procedure for SAM synthesis published by our lab, but using ethionine in place of methionine.[2] Briefly, 50 mM KCl, 26mM $MgCl_2$, 0.9 mM EDTA, 14.4 mM ATP, 12.0 mM L-ethionine, 1.14 mM 2-mercaptoethanol, and inorganic pyrophosphatase were combined in 100 mM Tris-HCl (pH 8.0), along with a crude lysate of SAM synthetase (1 mL), and incubated while stirring for 18 hours at room temperature. The reaction was quenched using 1 M HCl (1 mL), and then centrifuged immediately. The supernatant was loaded via a super-loop onto a 15S cation exchange resin in an HR 10/10 column (GE Healthcare). The SAE was eluted using a linear gradient from H_2O to 1 M HCl, with the SAE eluting at 0.45 M HCl. Solvent was removed from the collected SAE eluate by rotary evaporation. The SAE was resuspended in a glove box in 200 µL of 100 mM Tris pH 7.0, and the pH was adjusted to neutral. The SAE was aliquoted and flash frozen in $N_{2(l)}$ and stored at -80 ^{0}C. The absorbance of the SAE solution at 254 nm was measured and the concentration was calculated using an extinction coefficient of 15400 $M^{-1}cm^{-1}$. ^{1}H NMR (D_2O, 600 MHz): δ 8.48 (s, 1H), 8.47 (s, 1H), 6.17 (d, *J*=4.0, 1H), 4.83 (dd, *J*=5.3, 1H), 4.62 (dd, *J*=5.9, 1H), 4.56 (m, 1H), 4.11 (m, 2H), 3.98 (m, 2 H), 3.68-3.53 (m, 2H), 3.52-3.49 (q, 2H) , 2.51-2.35 (m, 2H), 1.45 (t, *J*=7.4, 3H). ESI-MS calculated m/z for $[C_{16}H_{25}N_6O_5S]^+$: 413.16, found 413.16.

HydG Activity Assay with SAM or Ethyl-SAM. Assay components for HydG-catalyzed CO formation were assembled in a Coy anaerobic chamber maintained with 97% N_2 and 3% H_2, and were carried out with modifications to our previously published protocol.[3] Reconstituted HydG samples were assayed for CO production using H64L deoxymyoglobin (deoxyMb) as a reporter.[4] Assays contained 20 µM HydG protein, 30 µM (heme) H64L Mb, 600 µM L-tyrosine, and 8 mM NaDT in 50 mM Tris, 10 mM KCl, pH 8.0 buffer, and were conducted at 37 °C in a 1 mm pathlength, anaerobic cuvette (Spectrocell Inc., Oreland, PA, USA). UV-visible absorbance spectra (250 – 800 nm) were recorded with a Cary 60 spectrophotometer (Varian) at 1.0 nm data intervals. Assays were initiated with the addition via a Hamilton gas tight syringe of either 2 mM SAM or 2 mM ethyl-SAM. The formation of CO and complexation by deoxyMb is evidenced by the shift in the Soret band (432 nm to 425 nm) and by the shift and splitting of the ≈565 nm band, which together give rise to a visible spectrum characteristic of MbCO. UV-Vis difference spectra were constructed from scans collected before and after addition of either SAM or SAE to the HydG, deoxyMb, tyrosine, and DT mixture; these difference spectra were used to correct for sample dilution and quantify the carboxy-Mb that was formed during the experiment. The $\Delta A_{425\ nm}$ and $\Delta\varepsilon_{425\ nm}$ were used to calculate MbCO concentration values at each time point, which were then used to determine apparent first-order rate constants, k, for the reactions.

Preparation of EPR samples. All samples for spectroscopy were prepared in either an anaerobic chamber (Coy Labs, Glass Lakes, MI) or an MBraun glove box, with oxygen levels maintained at 5-10 ppm and ≤ 0.5 ppm, respectively. HydG (1 mM) was chemically reduced by adding sodium dithionite (NaDT) to 3 mM final concentration. SAE was added to HydG to 5 mM final concentration, the mixture was transferred to EPR tubes, then samples were frozen and stored at 77 K until data acquisition.

EPR Measurements. EPR analyses were conducted using either a Bruker EMX spectrometer fitted with a ColdEdge (Sumitomo Cryogenics) 10 K waveguide in-cavity cryogen free system, with Oxford Mercury iTC controller unit and helium Stinger recirculating unit (Sumitomo Cryogenics, ColdEdge Technologies, Allentown, PA), or a Bruker ESP 300 spectrometer or a Bruker EMX spectrometer, both equipped with continuous helium flow Oxford cryostats.. The temperature was maintained at 12 K to record the iron-sulfur cluster spectra before and after photolysis, or 40 K to monitor radical species. The modulation amplitude (10 – 2 G) was varied to maximize resolution of signals. The modulation frequency was maintained at 100 kHz, while the power was varied depending on temperature to ensure non-saturating conditions were maintained. A 450 nm Thorlabs diode laser was employed to cryogenecially photolyze samples in the EPR cavity equipped with a waveguide-beyond-cutoff optical port.

Rapid Freeze Quench Sample Preparation. Rapid freeze-quench (RFQ) techniques were used to trap reaction intermediates at specific time intervals after synchronous initiation of the enzyme catalytic reaction. Samples were prepared in an anaerobic chamber (Coy Labs), with the oxygen maintained ≤ 5.0 ppm. A 50 μL aliquot of 1.0 mM HydG was chemically reduced with 3 mM (final concentration) NaDT, diluted to 100 μL using buffer (50 mM Tris pH 8.0, 250 mM KCl, 5% glycerol) in an Eppendorf tube, and allowed to incubate for at least 5 min. In a separate tube, *L*-tyrosine (6 mM) was mixed in the same buffer as the enzyme, along with 3 mM NaDT and either 5 mM SAM or SAE followed by a 5 min incubation. Each mixture was centrifuged to remove any debris that may be a resultant of precipitation. The samples were subsequently loaded into two separate syringes, which were then connected to the RFQ instrument. The enzyme and substrate were rapid-mixed and subsequently quenched after 500 ms, a time point that we previously found to provide strong signals for the Ω intermediate in HydG.[5] Final protein concentration in the RFQ samples was 250 μM.

Photolysis of Enzyme Complexes with SAE. In an anaerobic Coy chamber, HydG samples for *intra*-cavity photolysis and EPR spectroscopic analysis were prepared by mixing enzyme with Na-dithionite to reduce the clusters to the 1+ state, SAE, and buffer (50 mM Tris pH 8.0, 250 mM KCl, 10 % glycerol), to final concentrations of 100 μM or 200 μM protein, 3 mM DT, and 5.5 mM SAM. These samples were then briefly centrifuged, transferred to EPR tubes, flash frozen, and stored in liquid N_2; time from mixing to freezing was typically ~4 min. Yield of the ethyl radical on photolysis varied from 10% to 70%, depending on the time of photolysis as well as the size of EPR tube in which photolysis was done.

Results and Discussion

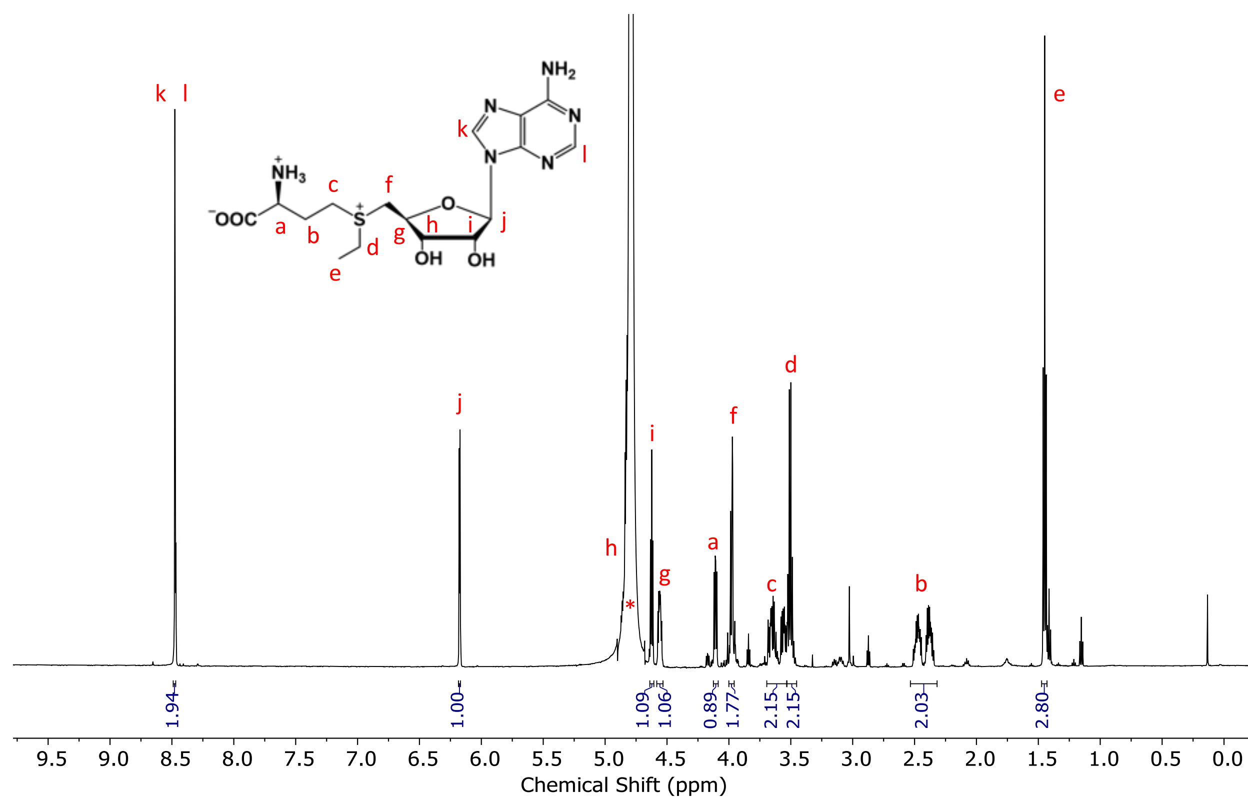

Figure S1. ^{1}H NMR spectrum of SAE acquired at 600 MHz. Sample preparation was achieved by resuspending SAE into D_2O immediately after purification and rotary evaporation (as described above), to a final concentration of ≈ 60 mM. Prior to NMR run, the SAE was kept frozen to minimize degradation. * Denotes residual solvent peak.

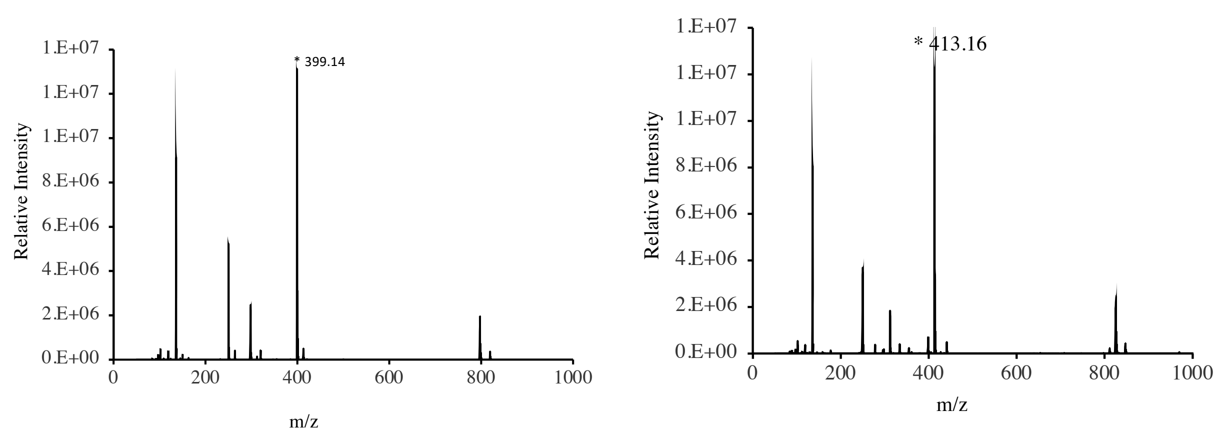

Figure S2. Mass spectrometry results of purified SAM (left) and SAE (right). Both compounds were resuspended in 50 mM Tris (pH 7.0) to ≈ 60 mM and 2 µL of each compound was injected into the LC-MS. The retention times of both SAM and SAE were similar (data not shown). MS analysis showed that ions with m/z of 399.14 and 413.16 m/z were present in the SAM and SAE samples, respectively. Agilent Qualitative Analysis B.06.00 of the MassHunter software suite was used to correctly assign peaks.

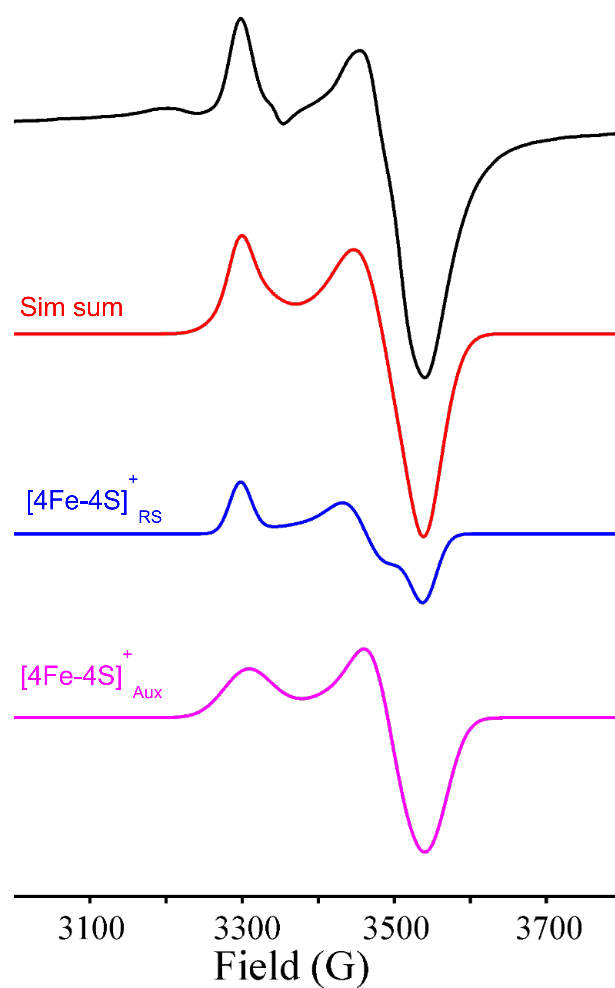

Figure S3. EPR spectral simulations for reduced HydG. The experimental data is shown in black. The simulation of the radical SAM [4Fe-4S] cluster ($[4Fe\text{-}4S]_{RS}$) is shown in blue, g = [2.031 1.935 1.893]. The simulation of the auxiliary cluster ($[4Fe\text{-}4S]_{AUX}$) is shown in pink, g = [2.027 1.919 1.888]. The sum of these simulations is shown in red.

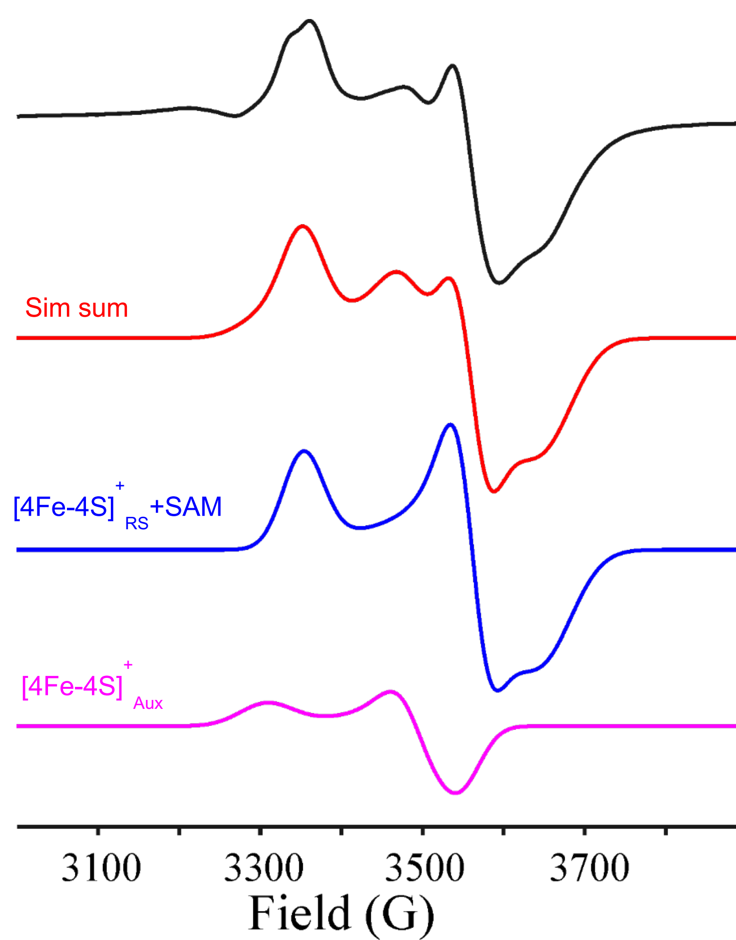

Figure S4. EPR spectral simulations for reduced HydG + SAM. The experimental data is shown in black. The simulation of the radical SAM [4Fe-4S] cluster with SAM bound ($[4Fe\text{-}4S]_{RS}$ +SAM) is shown in blue, g = [1.998 1.88 1.834]. The simulation of the auxiliary cluster ($[4Fe\text{-}4S]_{AUX}$) is shown in pink, g = [2.027 1.919 1.888]. The sum of these simulations is shown in red.

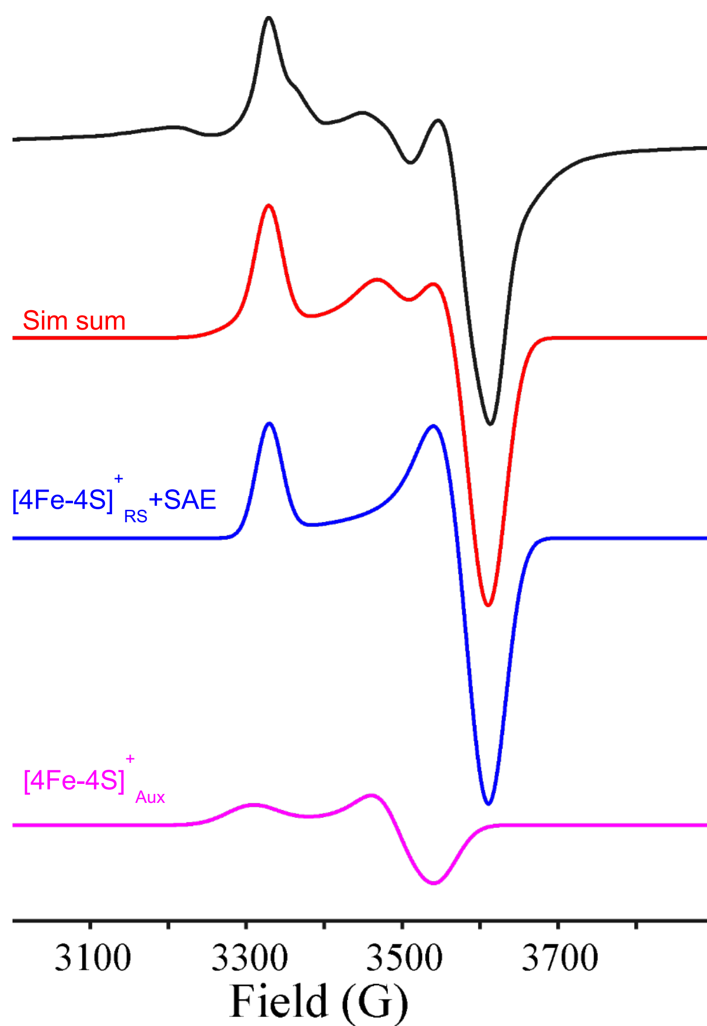

Figure S5. EPR spectral simulations for reduced HydG + SAE. The experimental data is shown in black. The simulation of the radical SAM [4Fe-4S] cluster with SAE bound ($[4Fe\text{-}4S]_{RS}$ + SAE) is shown in blue, g = [2.012 1.876 1.854]. The simulation of the auxiliary cluster ($[4Fe\text{-}4S]_{AUX}$) is shown in pink, g = [2.027 1.919. 1.888]. The sum of these simulations is shown in red.

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Author Contributions

Stella Impano: Preparation of samples, acquisition and analysis of data, lead contributor.

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Adrien Pagnier: Assisted in rapid freeze-quench sample preparation, supporting contributor.

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Brian Hoffman: Analysis of data, funding acquisition, lead contributor.

Joan Broderick: Project administration, funding acquisition, writing original draft, lead contributor.