Supporting Information

An Unusual Peroxo Intermediate of the Arylamine Oxygenase of the Chloramphenicol Biosynthetic Pathway

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EXPERIMENTAL PROCEDURES

Reagents. Bicine and other buffers used in this study were purchased from Fisher. ATP, *p*-aminophenol (pAP), *p*-aminobenzoate (pABA), and L-p-aminophenylalanine (L-pAPA) were purchased from Sigma-Aldrich. Tris-(2-carboxyethylphosphine) was purchased from Molecular Probes (Eugene, OR). ^{57}Fe (95.5 %) was purchased from Cambridge Isotope Laboratories (Andover, MA). Amino-chloramphenicol (D-threo-1-(4-aminophenyl)-2-dichloroacetylamino-1,3-propanediol hydrochloride) (NH_2-CAM) was synthesized by Toronto Research Chemicals, Inc.

Cloning and Heterologous Expression of CmlI. CmlI was cloned from genomic DNA isolated from *Streptomyces venezuelae* mycelia. The primers used for PCR amplification of CmlI are shown with restriction sites underlined:

Forward: CCATATGGCGATCGCCCGTGACCACACGGACGAGAAA

Reverse: CCATATGGTTTAAACTCATCGGGTCACCGTCGTGCC

PCR products were digested with AsiSI and PmeI and ligated to similarly digested pVP91A, obtained from the DNASU plasmid repository, to produce the N-terminal His_8 tagged enzyme. The final CmlI-containing plasmid was verified by sequencing at the University of Minnesota Biomedical Genomics Center. Expression of CmlI was performed in *E. coli* BL21(DE3) in M9 minimal medium in the presence of 100 μg/mL ampicillin. Cells were grown to an OD ~ 1.0 and induced with 150 μM IPTG and 50 μM $FeCl_3$ at which point the temperature was lowered to 20 °C, and growth was allowed to continue for an additional 15 h. Cells were harvested by centrifugation and stored at -70 °C until further use. ^{57}Fe enriched CmlI was prepared similarly from cells grown in M9 media with the addition ^{57}Fe metal dissolved in a minimal volume of *aqua regia* to a final concentration of 25 μM.

Purification of CmlI. Cells were resuspended in 50 mM potassium phosphate pH 7.4, 300 mM NaCl, 10 mM imidazole, lysed via sonication, and centrifuged. The resulting supernatant was loaded onto a Ni nitrilotriacetic acid column (Amersham) equilibrated in the same buffer. After loading, the column

was washed with the above buffer containing 25 mM imidazole, and protein was eluted in the same buffer containing 200 mM imidazole. Protein-containing fractions were pooled and dialyzed against 25 mM Bicine pH 9 and stored at -70 °C until further use. CmlI concentrations were determined by calculated extinction coefficient checked against denatured protein (ε_{280} = 50 mM^{-1} cm^{-1}).

CmlP NRPS. Construction, purification, phosphopantetheinylation, and L-pAPA loading of $CmlP_{AT}$, which contains the adenylation and thiolation domains of CmlP, were as previously described.[1] The quantitative loading of $CmlP_{AT}$ T-domain with L-pAPA was verified previously in LC-MS studies.[1]

Resonance Raman Studies. CmlI (2 mM) was reduced under anaerobic conditions with an excess amount of dithionite in the presence of 0.1 equivalent of methylviologen. Excess reductant and methylviologen were removed using a PD-10 desalting column (G-25, GE Healthcare) in an anaerobic chamber. The removal of methylviologen is essential as it can be photoreduced by the laser, which results in the reduction of the peroxo species and thus loss of rRaman signal. Aliquots of reduced CmlI from the same batch were exposed to either $^{16}O_2$ or $^{18}O_2$ at ~ 4 °C for several minutes. The samples were then snap-frozen with liquid nitrogen and kept at 77 K for future experiments. $^{16}O_2$ and $^{18}O_2$ samples were repeated three times. Multiple measurements were carried out for each sample. For each measurement, a ~ 70 μL aliquot of samples was thawed and transferred to flat-bottomed NMR quartz tubes and maintained at a temperature range of -10 °C to 10 °C. For the isotopically labeled samples, the tubes were sealed with septa and equilibrated with argon prior to sample transfer to minimize the exchange of the peroxo ligand with atmospheric dioxygen.

Resonance Raman experiments were performed on an Acton AM-506 spectrophotometer (1,200-groove grating) with a Princeton Instruments LN_CCD-1100-PB_UVAR detector cooled to -120 °C with liquid nitrogen. The 488 nm excitation line at 100 – 600 mW power was provided by a Spectra-Physics BeamLok 2060-KR-RS Argon ion laser, which is filtered out by a Kaiser Optical holographic super notch filter. Spectra were collected in 90° scattering geometry at resolution of 4 cm^{-1}, and referenced to indene.

Multiple spectra were continuously collected over 10 or 20 minutes with each spectrum obtained from a 15-second exposure. Spectra within desired time range were added to improve the signal-to-noise ratio. Multiple measurements were performed for each CmlI-peroxo sample. GRAMS/AI (Thermo Galactic, Salem, NH) was utilized data processing.

Analysis of CmlI Metabolites. A 1 mM solution of peroxo CmlI was prepared through the oxygenation of the dithionite reduced enzyme, as described above for the resonance Raman studies. The near stoichiometric conversion to the peroxo complex was verified by UV/Vis spectroscopy assuming an extinction coefficient of 500 M^{-1} cm^{-1} for the CmlI-peroxo intermediate at 500 nm (determined via spectral analysis of samples prepared for Mössbauer studies). NH_2-CAM (dissolved in methanol) was added to the enzyme at a final concentration of 10 mM and allowed to incubate for 1 min at 4 °C. The samples were subsequently quenched with the addition TCA to a final concentration of 1% (vol/vol). Precipitated protein was removed by centrifugation for 10 minutes at 4 °C at 14,000 rpm in a microcentrifuge. Alternatively, samples were filtered using 10K Amicon Ultra centrifugal filters. LC-MS analysis was performed on a Hewlett Packard 1100LC using a Waters Acquity C18 column (2.1 × 50 mm × 1.7 μm) maintained at 35 °C with a gradient elution of 3 to 97% acetonitrile in 0.1% formic acid over 8 minutes at a flow rate of 0.40 mL⁄ min. Mass monitoring was performed using a Bruker Microtof orthogonal quadrupole time of flight mass spectrometer scanning from 50 to 1000 m⁄z. HPLC analysis was performed on a Waters system with a 1525 binary pump, 2487 dual wavelength UV/Vis detector, and Aglient Zorbax SB C18 column (2.6 × 150 × 5 μm) with a gradient of 4 to 100% acetonitrile and 0.1% formic acid over 7.5 minutes after 2.5 minutes of isocratic flow at 4% acetonitrile 0.1% formic acid. The detection wavelength for CAM, pABA and all related compounds was 280 nm and 305 nm for pAP and its related compounds.

Metal Analysis. CmlI quantified by 280 nm absorbance was treated in 4 M nitric acid for 2 hours. The denatured sample was centrifuged at 25,000 x g for 20 min and the supernatant transferred to a

volumetric flask for dilution to 5 ml with dH_2O. The metals present in the sample were quantitated using inductively-coupled plasma mass-spectrometry (ICP-MS) at the UMN Earth Sciences Department.

Stopped Flow Studies. Stopped-flow experiments were performed using an Applied Photophysics model SX.18MV stopped flow device. All stopped flow experiments were performed in 50 mM Bicine pH 9 at 4 °C. In reactions of the diferrous enzyme with O_2 and arylamine substrates, CmlI was reduced in a Coy anaerobic chamber with a 3-fold excess of dithionite in the presence of 25 μM methyl viologen. Excess dithionite and methyl viologen were removed through chromatography on a PD-10 desalting column (GE Healthcare) and the reduced protein was loaded anaerobically using a Hamilton syringe to the stopped-flow device which had been previously scrubbed with dithionite and anaerobic buffer. Reactions of the pre-formed CmlI-peroxo complex with arylamine substrates were performed similarly, with the diferrous enzyme extensively oxygenated prior to loading the stopped flow syringe. The kinetic data were analyzed to extract reciprocal relaxation times using the Applied Photophysics ProData Viewer program. Reaction time courses were fit to summed exponential expressions, which are appropriate for a series of first order or pseudo-first order reactions.

Titration of CmlI-peroxo with NH_2-CAM. CmlI-peroxo was prepared as described above at 500 μM concentration and stored in a quartz cuvette on ice. Small aliquots of a 1 mM NH_2-CAM solution in 50 mM Bicine buffer, pH 9 were added each representing approximately 0.05 equivalents relative to the starting CmlI-peroxo concentration. The optical spectrum was scanned and used to construct the plot in Figure 3, *inset*. About 3 % of the CmlI-peroxo decays spontaneously during the experiment. No correction was made for this loss. The end point of the reaction was analyzed by reverse phase HPLC and an authentic standard of CAM was run by the same HPLC method to confirm that the UV-Vis titration was producing CAM.

EPR and Mössbauer. EPR spectra were collected using a Bruker Elexsys E-500 spectrometer equipped with a Bruker dual mode cavity and an Oxford ESR 910 liquid helium cryostat. Mixed valent CmlI

was prepared via photoreduction of the diferric enzyme (1 mM) at 4 °C with 50 μM 5-deazaflavin in the presence of 10 mM phenazine methosulfate using a standard 100 W lamp. Mössbauer spectroscopy was performed as previously described.[1] Spectra were analyzed using the software WMOSS (SEE Co., Edina, MN).

SUPPLEMENTARY FIGURES

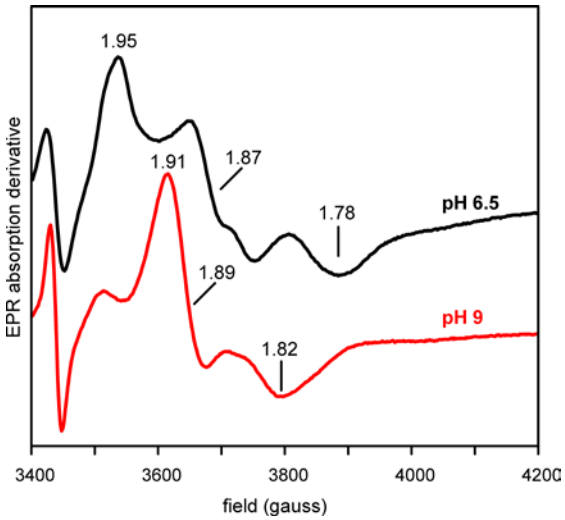

Figure S1. EPR spectra of mixed valent CmII produced by photoreduction at pH 6.5 (top, black) and pH 9.0 (bottom, red), recorded at 15 K (black) and 45 K, respectively, at a microwave power of 10 mW.

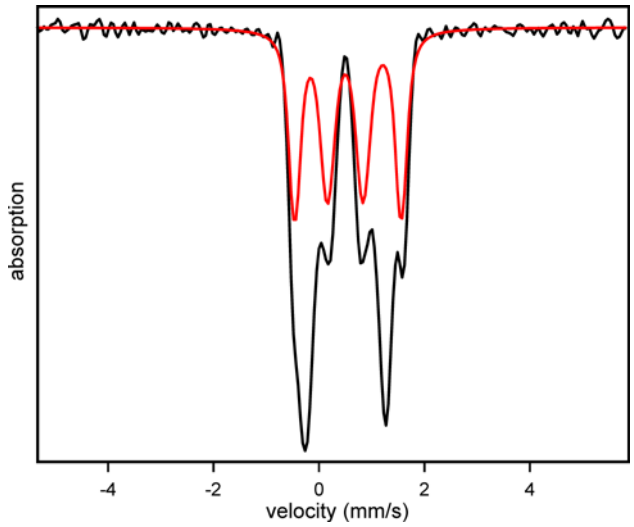

Figure S2. Fourier Transform treated Mössbauer spectrum of diferric CmlI at pH 9.0. A Mössbauer spectrum is the convolution of the spectrum of the absorber with the spectrum emitted by the ^{57}Co Mössbauer source. In our Fourier transform treatment, the experimental spectrum is transformed into the time-domain. The known source contribution to the linewidth is then removed in the time domain. The figure shows the back-transformed spectrum. The red line indicates the nested doublets 1 and 4. Doublets 2 and 3 contribute to the most intense peaks.

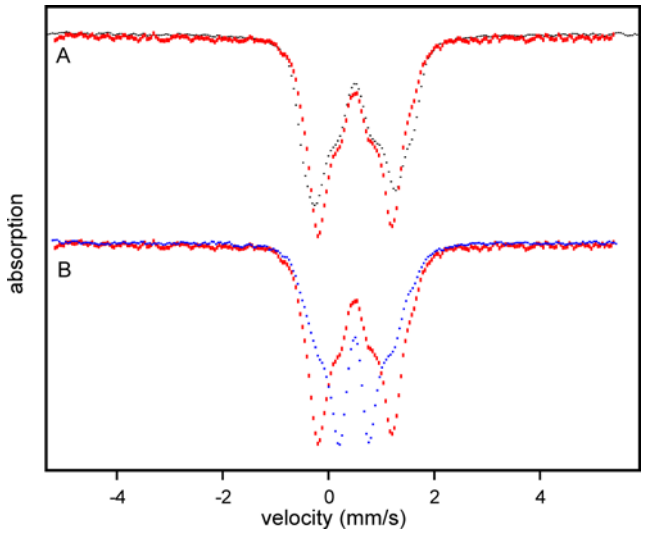

Figure S3: Comparison of the 4.2 K Mössbauer spectra of diferric CmlI prepared at different pH values. (A): Spectra recorded at pH 9 (black) and pH 7.5 (red). (B): pH 7.5 spectrum (red) is shown together with the pH 6.5 spectrum (blue).

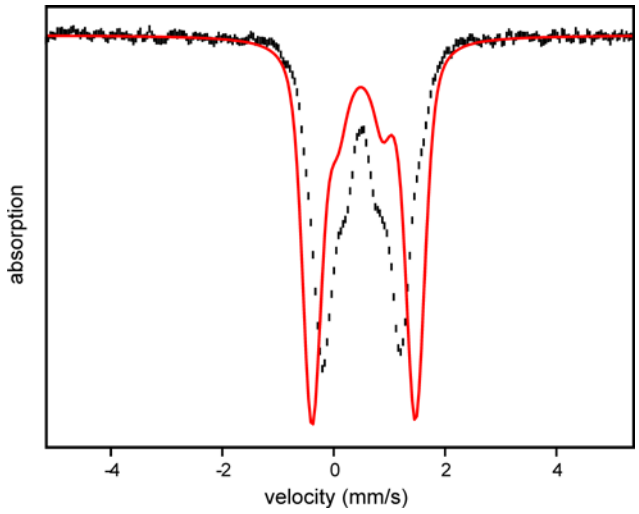

Figure S4. Comparison of the pH 7.5 spectra of CmlI (black) and AurF (red). The AurF spectrum is our simulation based on inspection of Figure S1 of Li et al.[2]

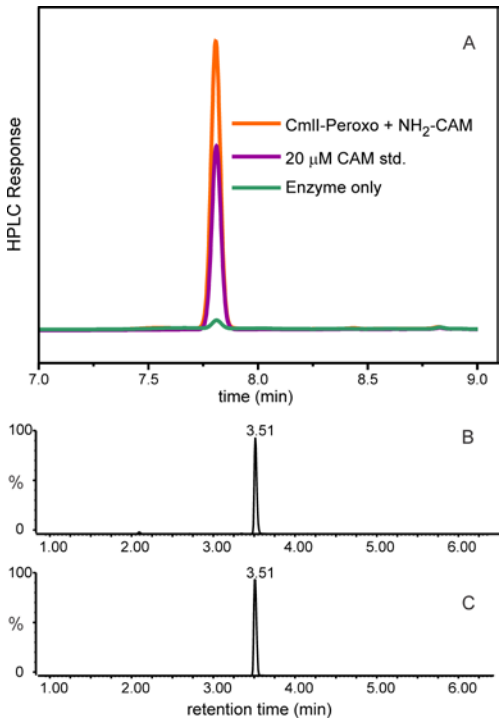

Figure S5. Product from reaction of 1 mM CmlI-peroxo with 10 mM NH_2-CAM. Top: (A) HPLC traces of the extracted reaction mixture at the end of the titration in comparison to an authentic chloramphenicol standard. Bottom: Extracted ion chromatograms at m/z = 321 for the reaction product (B) and a chloramphenicol standard (C). Buffer: 50 mM Bicine, pH 9.0.

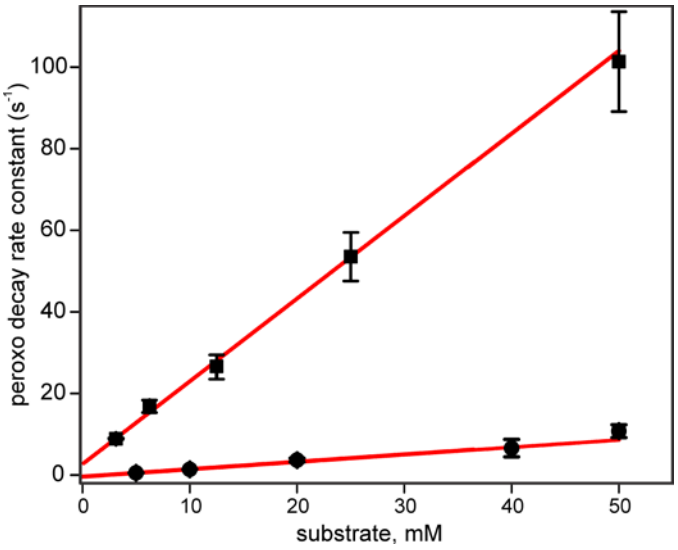

Figure S6. Linear dependence of CmlI-peroxo decay rate with L-pAPA (squares) and pABA (circles). In these experiments, the CmlI-peroxo was pre-formed and loaded into a stopped flow syringe. The substrates were mixed in large excess over the enzyme from the second syringe. This method results in a single observed exponential kinetic phase which can be fit with nonlinear regression to give the observed rate constants.

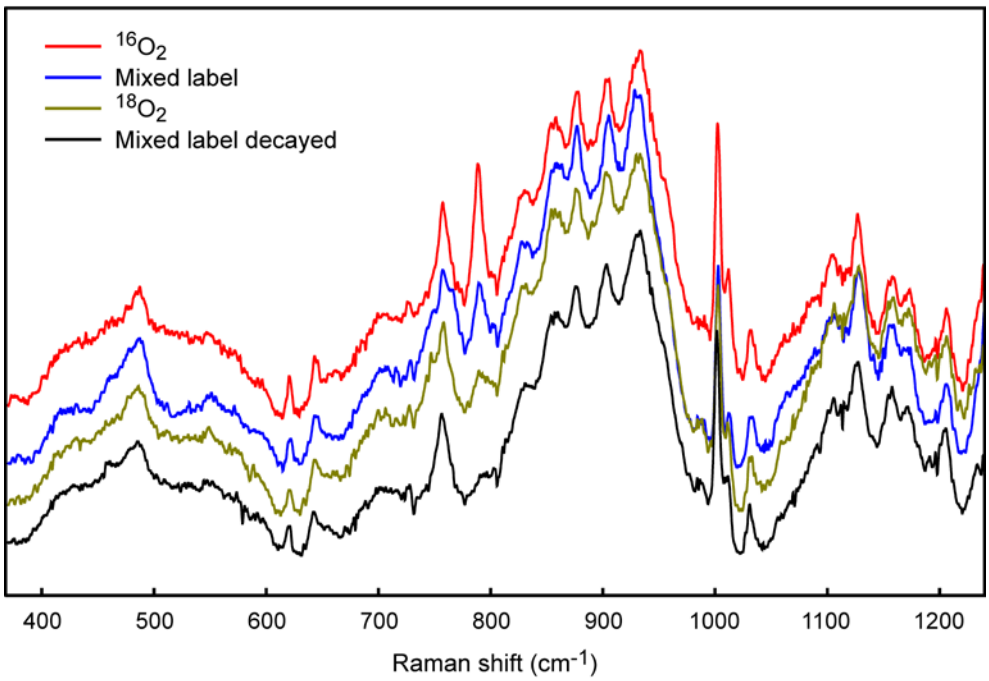

Figure S7. Resonance Raman spectra of CmlI-peroxo (from top to bottom): $^{16}O_2$; mixed labeled O_2, $^{18}O_2$; $^{18}O_2$; and decayed CmlA-peroxo. Protein vibrations obscure the region between 840 and 980 cm^{-1} omitted from Figure 6.

SUPPLEMENTARY REFERENCES

(1) Makris, T. M.; Chakrabarti, M.; Münck, E.; Lipscomb, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 15391.

(2) Li, N.; Korboukh, V. K.; Krebs, C.; Bollinger, J. M., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 15722.