Supporting Information

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SI Text

Supporting Information Available. Additional experimental details regarding: (*i*) reagents; (*ii*) the preparation of isotopically labeled $NaCl^{18}O_2$; (*iii*) the determination of Cld halogenase activity; (*iv*) monitoring dioxygen evolution in oxygen-18 labeled water and from oxygen-18 labeled chlorite; (*v*) stopped-flow UV-vis spectrophotometry; (vi) electron paramagnetic resonance.

Reagents. Buffer reagents were purchased from Fisher and prepared by using deionized Millipore Milli-Q water. 95% oxygen-18-enriched water was obtained from Cambridge Isotopes. $NaClO_2$ (GFS), NaClO, $Ca(ClO)_2$ (Sigma-Aldrich), $NaClO_3$ (GFS), PAA (Sigma-Aldrich), thioanisole (Acros) and L-ascorbic acid (Sigma-Aldrich) were used as obtained. Isotopically labeled $NaCl^{18}O_2$ (77% ^{18}O-enriched) was prepared from enriched chlorate (1). Recombinant chlorite dismutase from *Dechloromonas aromatica* RCB was prepared as reported (2).

Preparation of Isotopically Labeled $NaCl^{18}O_2$. Isotopically labeled chlorite was prepared and used *in situ* based on a modified protocol for the production of sodium chlorite from chlorate (1). In a thick-walled Schlenk tube, 2.1 g (20 mmol) of $NaClO_3$ was stirred in 5 ml of 95% enriched $H_2{}^{18}O$. To this mixture, 0.83 ml of concentrated sulfuric acid was added, and the mixture was capped and stirred for 1 h at 70°C. After this period, the tube was removed from heat, cooled to room temperature, and frozen in liquid N_2. The tube was opened to air, and 1.1 g (9 mmol) of solid Na_2SO_3 was added to the frozen mixture. The tube was sealed and warmed to melt the solution, and the mixture was placed back in the heating bath and stirred for 1.5 h in the dark to afford the appearance of yellow ClO_2 gas. This gas was bubbled through a ice-cold solution of NaOH (9 mmol), 1.1 ml of 30% H_2O_2 (9.7 mmol) and 2 ml of H_2O. The resulting solution was stirred with 0.5 g of MnO_2 to disproportionate unreacted H_2O_2 for 2 h. The solution was filtered and titrated to neutrality with 3 M H_3PO_4 (typically 1–1.5 ml). The resulting solution is 77% ^{18}O-enriched ClO_2^- in phosphate buffer (0.6 M).

Halogenase Activity Assay. Cld at 2 μM (heme) was incubated in the presence of 10 mM NaCl, NaBr, or NaI in a 100 mM phosphate buffer (pH 6.8) or a citrate phosphate buffer (pH 4.0). Either PAA (10 eq), peroxide (10 eq), or chlorite (final concentration, 50–600 μM) was then added and the solution mixed via pipet. Disappearance of the peak at 290 nm resulting from conversion of MCD (100 μM) to DCD was monitored (ε = 17.7 mM^{-1} cm^{-1}) (3). Control experiments using either HRP or no enzyme were carried out in parallel. In the absence of enzyme, PAA is capable of uncatalyzed bromination or iodination of MCD; H_2O_2 was therefore used as the oxidant in these reactions.

Monitoring Dioxygen Evolution in Oxygen-18-Labeled water and from Oxygen-18-Labeled Chlorite. Evolved oxygen was analyzed by using an in-house built mass spectrometer. This technique requires a substrate concentration high enough to produce at least 50 μl of oxygen for a reliable response. Typically, 900 μl of freshly prepared $NaClO_2$ (final 50 mM) in 100 mM sodium phosphate buffer, pH 7, was stirred in a septum-sealed glass cell with minimal head space. The reaction was started by a syringe injection of 100 μl of Cld (85 nM). The substrate, enzyme, and buffer were prepared in 95% oxygen-18-enriched water. An inert carrier gas (N_2) was drawn over the reaction head space at 2 ml/min by a Varian model SH 100 vacuum pump, carrying the evolved oxygen from the reaction to a mass spectrometer (Stanford Research Systems RGA 100, equipped with an Alcatel ATH31 Series turbopump). Mass spectra were collected until oxygen evolution ceased ($\approx$60 min). Analogous procedures were used in the presence of $Cl^{18}O_2^-$ and unlabeled water.

Stopped-Flow UV-vis Spectrophotometry. Intermediates were detected after addition of 20 eq of PAA or ClO^- to $\approx$10 μM Cld (in 0.1M sodium phosphate buffer, pH 7). Rapid repeat scans were performed at 10°C by using an Applied Photophysics SX.18MV Stopped-Flow Analyzer equipped with a 1.00-cm path length cell. The kinetics of compound I and II formation were monitored at 400 nm and 415 nm, respectively, in the single-mixing operation mode. Kinetic traces and second-order plots were fitted by using Kaleidagraph v3.6 for PC. The sequential multimixing mode was used to monitor reduction of compounds I and II with thioanisole and ascorbate, respectively. Thioanisole (1,500 eq) was mixed with the intermediate formed after a delay time of 10 ms (compound I) from the initial mixing of 10 μM Cld with 20 eq of PAA. For ascorbate, 600 eq were mixed with the intermediate formed after a delay time of 20 ms (compound II).

Electron Paramagnetic Resonance. Spectra were acquired on a Bruker EMX spectrometer equipped with an ER 4116DM Dual Mode X-band Resonator, an ER 082C magnet power supply, an Oxford Instruments ITC4 helium cryostat, and an Oxford Instruments VC40 flow controller. Mod. Frequency = 100 kHz, Mod. Amplitude = 6 G, MW frequency = 9.65 GHz, mW power = 50.24 mW. Spectra were scanned from 500 to 4,500 G. Solutions were prepared in 20% ethylene glycol, 80% 0.010 M phosphate buffer (pH 7.14). For observation of Cld under turnover conditions, 75 μl of a 17 μM solution of enzyme at 4°C was placed into an EPR tube and diluted with 125 μl of a 1.7 M solution of $NaClO_2$ at 4°C. The resulting reaction quickly foams; immediately after addition of ClO_2^-, the tube is flash frozen in liquid N_2. The resulting solid foam was analyzed by EPR. For warming, the tube was removed from the instrument and warmed at room temperature until the foam at the edge of the tube began melting. The tube was flash frozen again in liquid N_2 and analyzed.

1. Zhou C, Long J Xu P (1993) Preparation, properties and application of sodium chlorite and chlorine dioxide. *Huaxue Shijie* 34:38–40.
2. Streit BR, DuBois JL (2008) Chemical and steady state kinetic analyses of a heterologously expressed heme dependent chlorite dismutase. *Biochemistry* 47:5271–5280.
3. Hewson WD, Hager LP (1979) Mechanism of the chlorination reaction catalyzed by horseradish-peroxidase with chlorite. *J Biol Chem* 254:3175–3181.

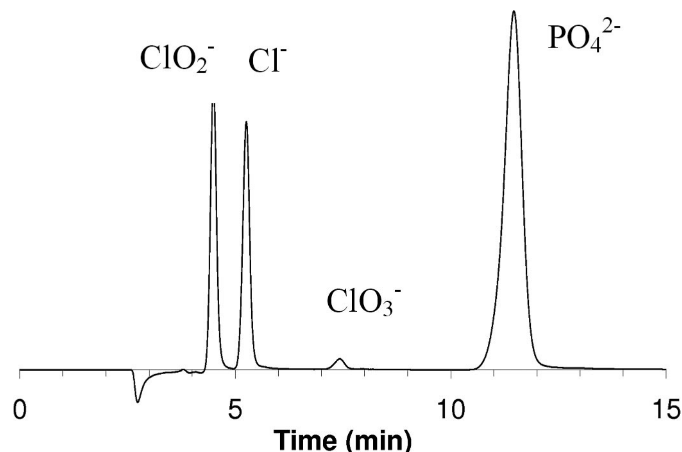


Fig. S1. Ion chromatogram of Cld dismutation products from 100-fold dilution of enzyme reaction mixture (85 nM Cld, 50 mM chlorite, 0.1 M sodium phosphate buffer, pH 7). Retention times for chlorite, chloride, chlorate, and phosphate are 4.48, 5.25, 7.42, and 11.47 min, respectively. Note that a small amount of ClO_3^- was present in the starting material (see Table S1). Na_2CO_3 (9 mM) was used as eluant. Chromatography calibration standards were 0.05–60 mM. O_2 was quantified by using a Chemglass 1818 Gas Evolution Measurement Apparatus. A typical Cld quantitation reaction is as follows: 9 ml of a 55.5 mM solution of $NaClO_2$ in 0.1 M sodium phosphate buffer was placed into a Schlenk tube and attached via tygon tubing to the gas evolution measurement apparatus. The pressure was equalized, and the measurement tube sealed from atmosphere. Stock enzyme solution (8.5×10^{-5} mM) was diluted 100-fold to 1 ml with 0.1 M sodium phosphate buffer and added to the Schlenk tube. The reaction was stirred for 1 h, after which the O_2 yield was measured on the graduations, correcting for the 1-ml injection volume. The resulting solution was diluted 100-fold with water and analyzed by ion chromatography. Chlorite stock solutions used in the above experiments were analyzed via ion chromatography in comparison to standard curves. Final chloride and chlorite concentrations were corrected by subtracting the concentrations of these species present in solution before reaction.

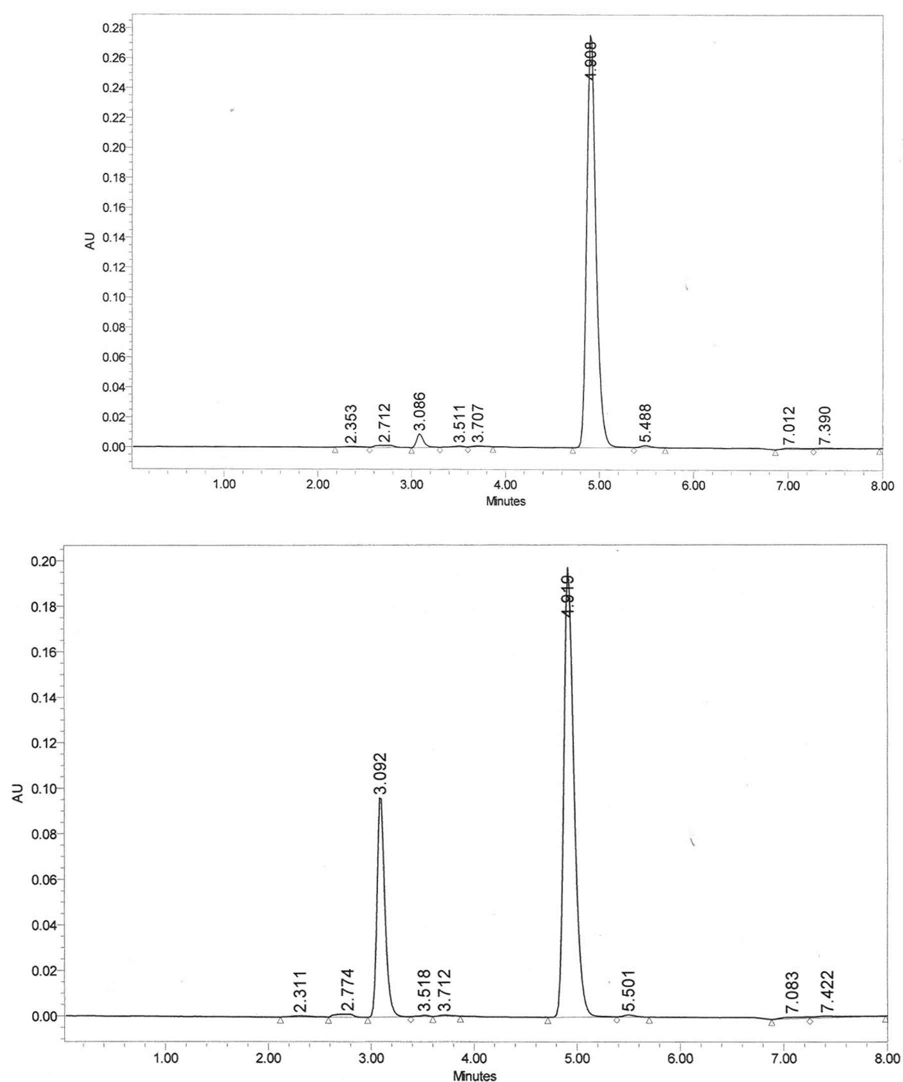


Fig. S2. Typical HPLC traces of extracted reactions of Cld with PAA and thioanisole at $\lambda = 341$ nm. Peak at 3.1 min corresponds to the oxidized product methyl phenyl sulfoxide and the peak at 4.9 min corresponds to thioanisole. Upper trace shows oxidation of thioanisole by Cld with 20,000 eq of ClO_2^- as the oxidant and the lower trace shows oxidation of thioansole by Cld with 100 eq of PAA, both as described in *Methods*. Concentrations of reactant and product were determined by comparison with standard curves (10–1,000 μM) of thioanisole and phenyl methyl sulfoxide, respectively. Samples were analyzed by HPLC by using a Phenomenex Hypersil 5 μm ODS (C18) column, 250 $\times$ 4.6 mm, on a Waters Alliance 2695 Separations Module equipped with a Water 2996 Photodiode Array detector. Mobile phases were: A, water + 0.5% formic acid; B, CH_3OH + 0.5% formic acid. Unreacted thioanisole and the methylsulfinylbenzene product were separated isocratically in 20% A/80% B within 8 min. UV-vis detection was performed in the range of 210–400 nm, with the data extracted at 341 nm. Reactions were compared with no-enzyme control samples.

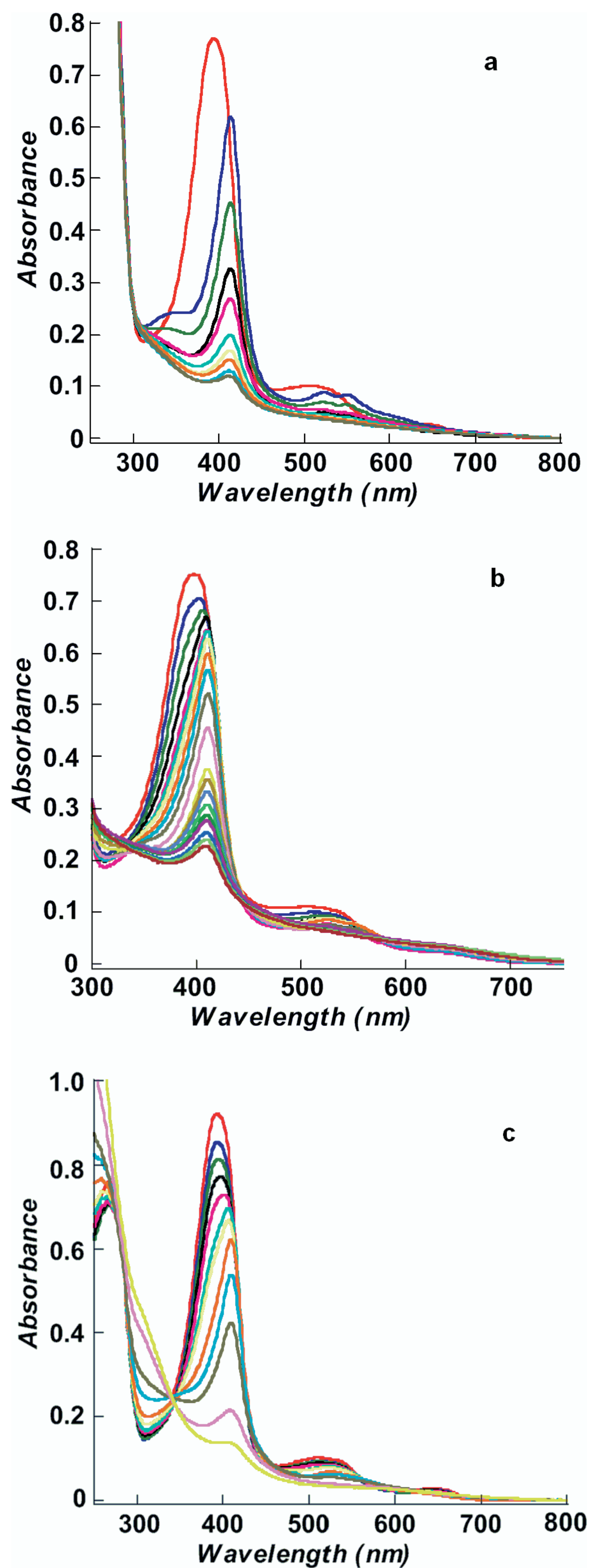


Fig. S3. Titration of Cld heme spectrum with oxidants. Samples were 8–10 μM in Cld-heme. In each case, the ferric species is oxidized to a ferryl-oxo (compound II-like) species with a red-shifted Soret absorbance and split peaks in the visible region. Spectra were measured after successive additions of aliquots of oxidant: (*a*) 10, 20, 30, 40, 50, 60, 70, 80, 90 eq of peracetic acid; (*b*) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90,100 eq of hypochlorite; (*c*) 1, 2, 4, 6, 8, 10, 14, 20, 30, 50, 70 eq of *m*CPBA (*m*-chloroperbenzoic acid). In each case, the concentration of the oxidant was determined via iodometric titration. All measurements were at 9°C in a 100 mM phosphate buffer, pH 6.8.

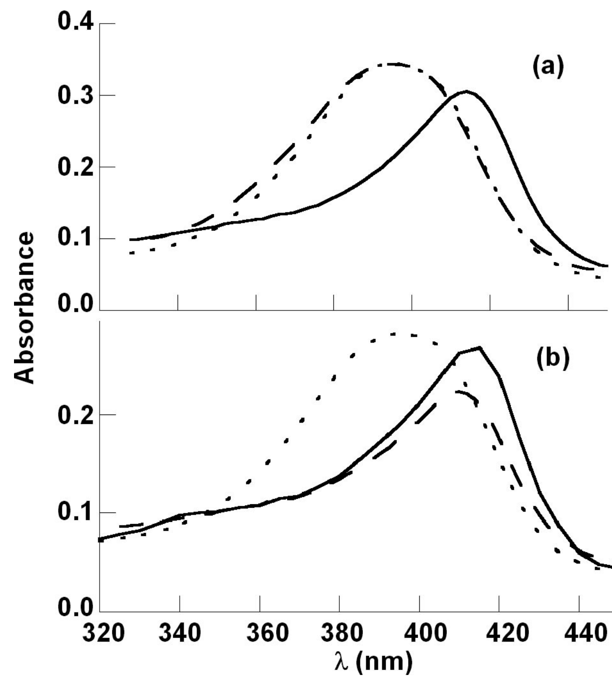


Fig. S4. Double-mixing experiments showing reaction of Cld intermediates with 1e$^-$ and 2e$^-$ substrates. (*a*) Compound II (solid line) reduction back to ferric enzyme by 600 eq of ascorbate (dashed line), which was added 20 ms after mixing 10 μM Cld (final concentration, 5 μM) with 20 eq PAA. The spectrum of the ferric resting enzyme without oxidant (dotted line) is included for comparison. (*b*) Compound I generated 10 ms after rapid mixing of 20 eq PAA with 10 μM Cld (final concentration 5 μM) was reacted with a second syringe containing 1500 equivalents of thioanisole. The resulting product spectrum (dashed line) is composed of 30% ferric resting state (dotted line) and 70% Compound II (solid line).

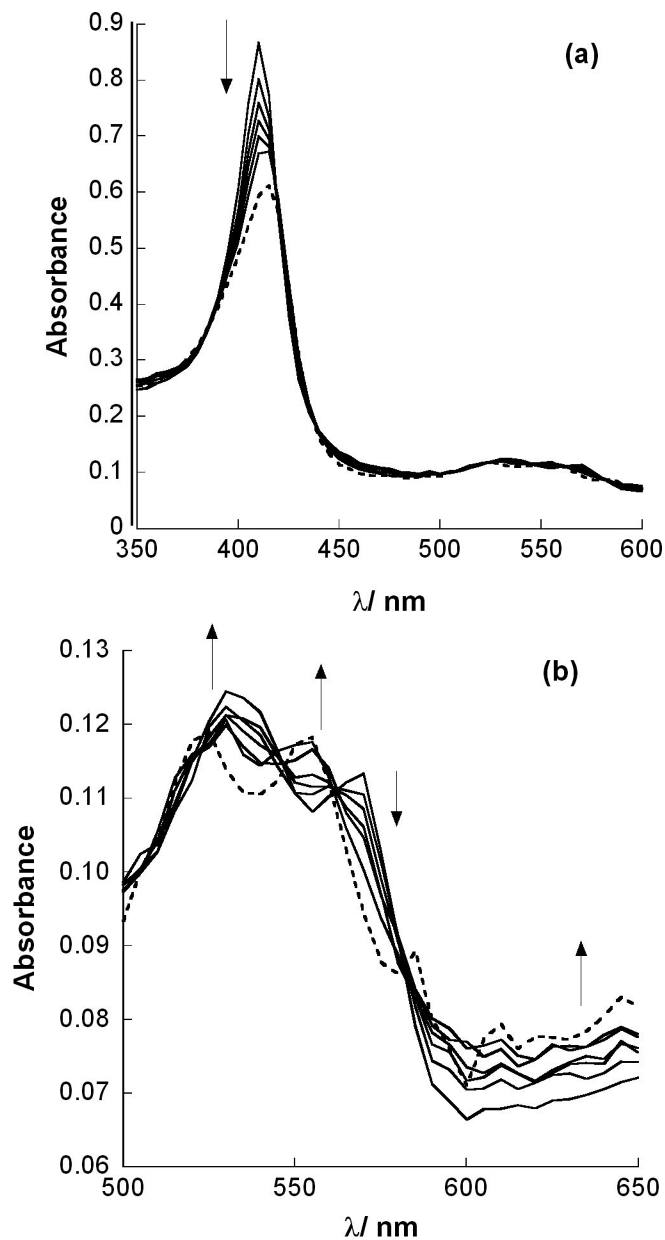


Fig. S5. Spectral changes within the first 1 s after 10 μM Cld was mixed with 20 eq of ClO^-. Spectra were measured at 3, 8, 13, 18, 25, and 38 ms (solid lines), and 1,250 ms (dashed lines). The final spectrum has peak energies indicative of compound II. (*a*) Soret region; (*b*) visible region on an expanded scale.

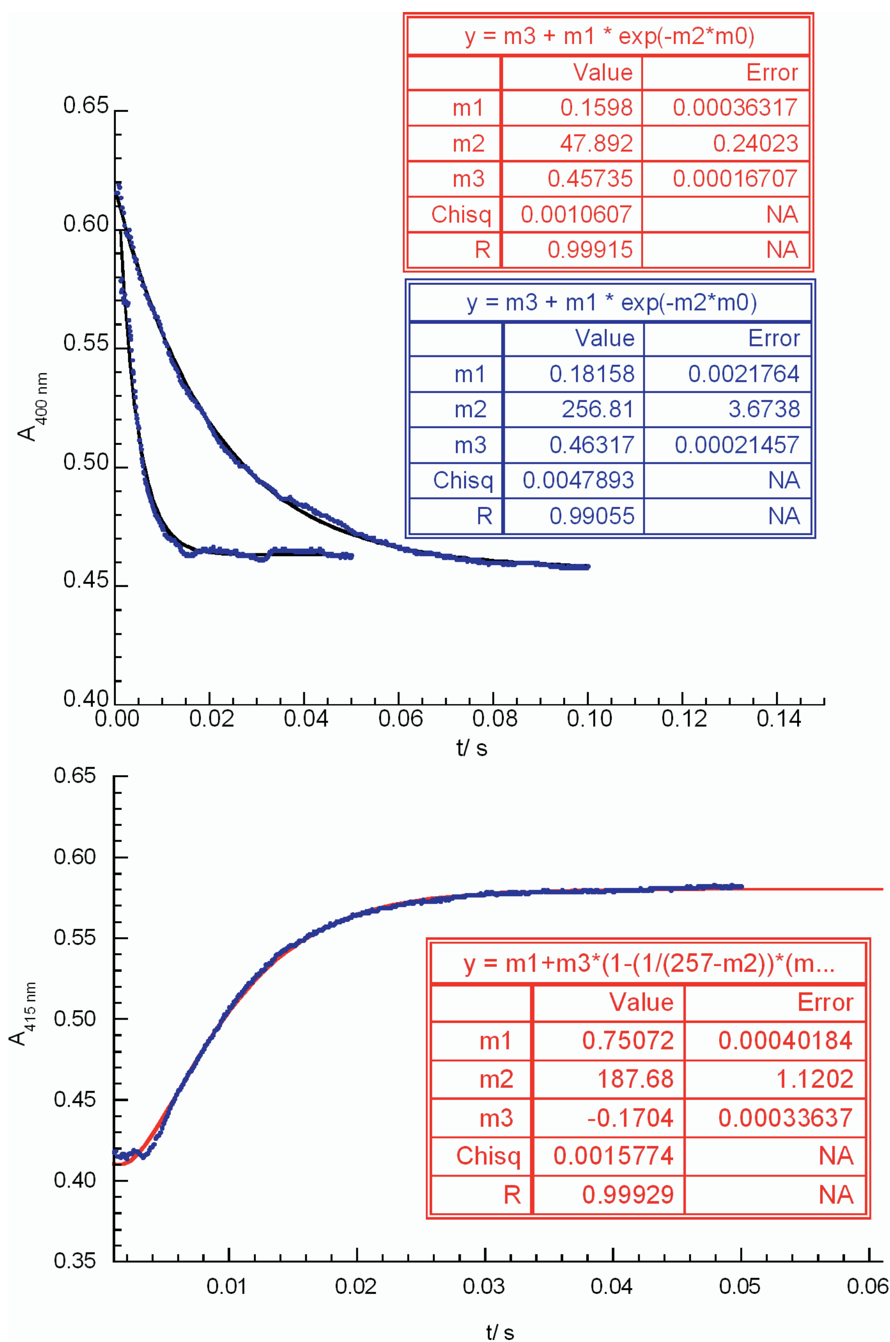


Fig. S6. Typical kinetic traces and fits (red or black lines) to the raw data (blue dots). (*Upper*) Compound I formation followed at 400 nm with 50 and 200 mM PAA. The data are fitted to pseudo-first-order rate law. (*Lower*) Formation of compound II followed at 415 nm with 200 mM PAA. The data are fitted to Eq. **3** in the main article.

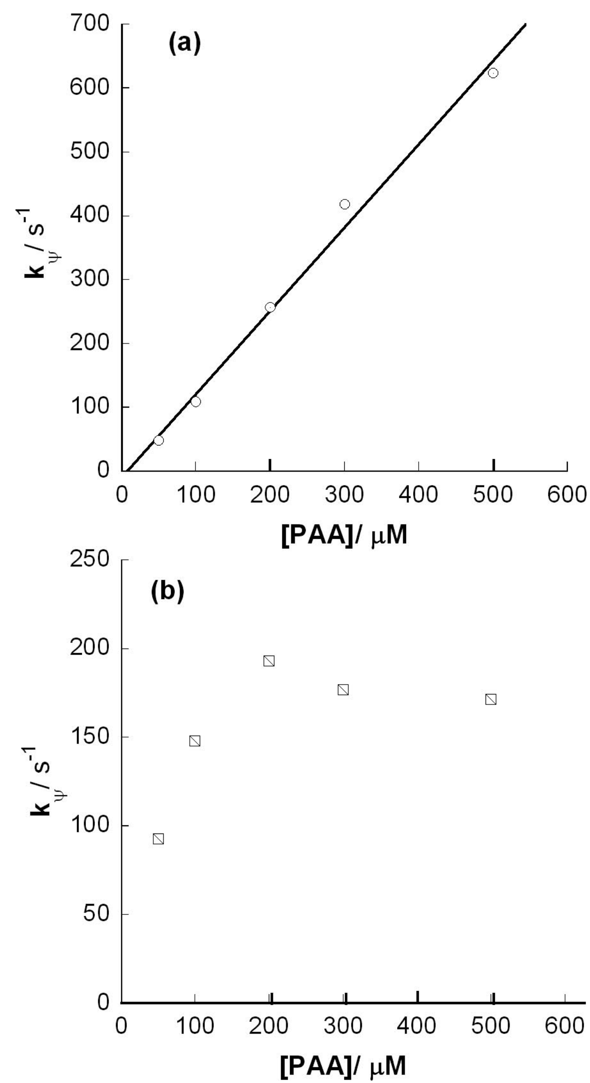


Fig. S7. Kinetics of ferryl intermediates formation from the reaction 10 μM Cld with excess PAA at 10°C. (*a*) Compound I formation: plot of pseudofirst-order rate constants vs. [PAA]. Pseudofirst-order rate constants were obtained by fitting kinetic traces monitored at 400 nm. (*b*) Compound II formation: plot of pseudo-first-order rate constants vs. [PAA].

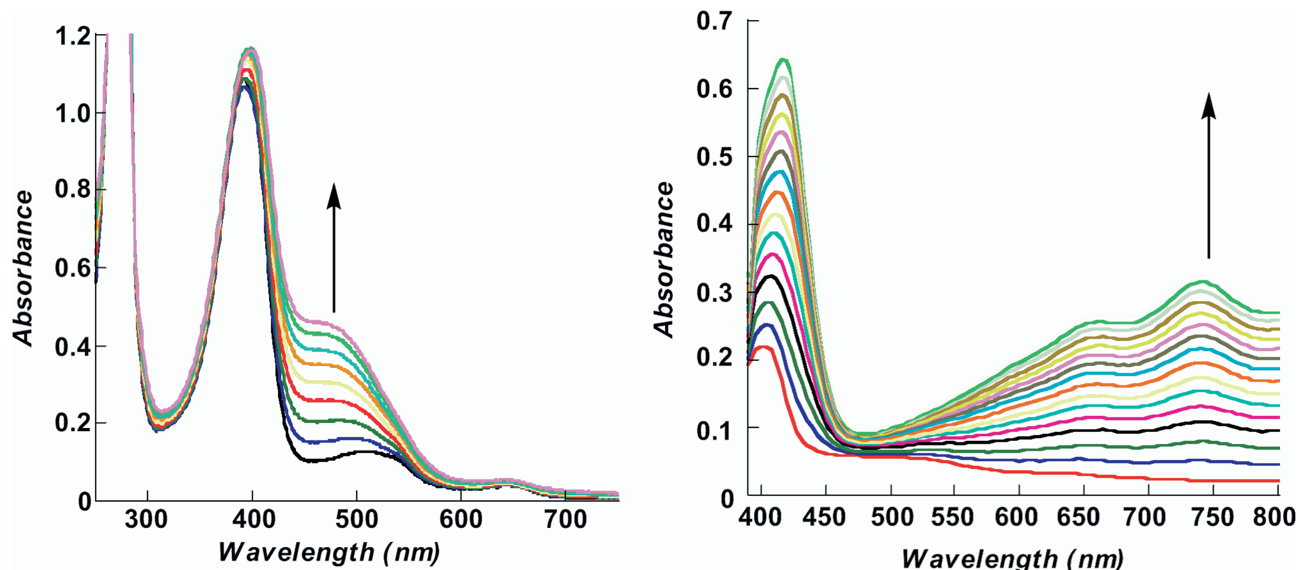
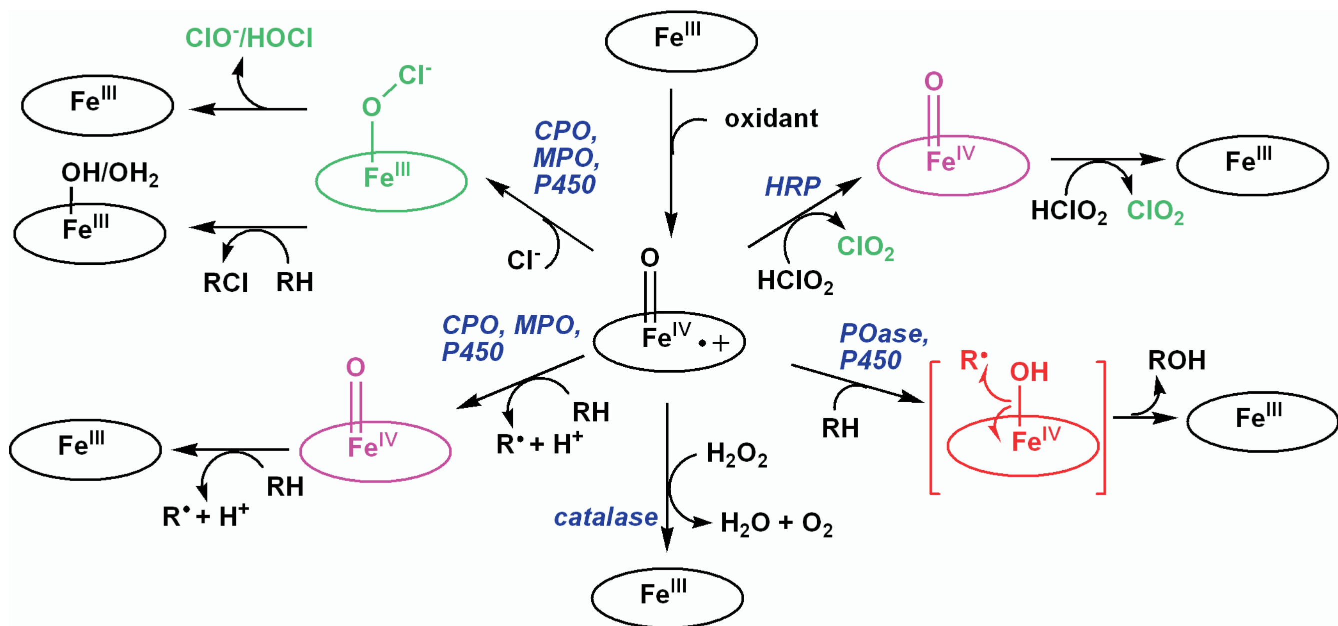


Fig. S8. Reaction of successive aliquots of 5 eq PAA and Cld in excess guaiacol (*Left*) and ABTS (*Right*) (1 mM guaiacol/ABTS). (*Left*) 11 μM Cld-associated heme; (*Right*) 2.7 μM heme. Addition of 1–3 μl of 14.4 mg/ml enzyme stock (3.3 heme per protein) to 147 μl of 100 mM phosphate buffer (pH 6.8) in a cuvette yielded samples 2.7–8.1 μM in heme. One microliter of a 90 mM guaiacol or ABTS solution (final, 600 μM) was added and a spectrum taken. Aliquots of 1–5 heme eq of PAA or 2,000 eq of chlorite were then added to the cuvette in 1- to 5-μl volumes and a spectrum measured at 25°C in a Pelter-thermostated Cary 50 spectrophotometer (used for all measurements in this study) after each addition. The stoichiometry of reaction after each addition was determined by using $\varepsilon = 2.66 \times 10^4\ M^{-1}\ cm^{-1}$ (470 nm) for guaiacol and $1.4 \times 10^4\ M^{-1}\ cm^{-1}$ (730 nm) (which does not overlap with the heme spectrum) for ABTS [Matsui T, Ozaki S, Watanabe Y (1999) Formation and catalytic roles of compound I in the hydrogen peroxide-dependent oxidations by His64 myoglobin mutants. *J Am Chem Soc* 121:9952–9957]. Control experiments using HRP, no enzyme, or 1 μM $FeCl_3$ were conducted in parallel.



Scheme S1. Partial summary of reactions catalyzed by heme peroxidases and related enzymes. The natural oxidant is typically H_2O_2, but can be chlorite or a peracid. One-electron oxidants are shown in purple. Chlorinating agents are green. The reactive hydroxylating species of cytochrome P450 (axial ligand = cysteine) or peroxygenases (axial ligand = histidine) is in red. Legend: CPO, chloroperoxidase; MPO, myeloperoxidase; P450, cytochrome P450; HRP, horseradish peroxidase; POase, peroxygenase.

Table S1. Recovery results from reaction of 10 ml of 50 mM ClO_2^- with 10 μl of 8.5 × 10^{-5} M Cld

Compound	C_i*	C_∞†	Yield/recovery‡	Yield/recovery, %
O_2	–	4.6 ml	4.6 ml	41
Cl^-	2.8 mM	25.7 mM	22.9 mM	49
ClO_3^-	1.6 mM	1.4 mM	0 mM	0
ClO_2^-	46.7 mM	26.4 mM	26.4 mM	51

*Represents the amount present before enzyme addition.
†Total amount present after reaction completion (ca. 60 min).
‡$C_\infty - C_i$. This subtraction is not necessary for O_2 yield and ClO_2^- recovery.

Table S2. Analysis of products of reaction of Cld, oxidants, and oxidizable substrates

[heme Fe], μM*	Substrate†; oxidant	Oxidant equivalents‡	Product:oxidant§	Assay method
2.7–8.1	ABTS, PAA	1–5	1.6 ± 0.1	UV-vis
8.1	ABTS, ClO_2^-	20,000	0	UV-vis
1.4	Thioanisole, PAA	100	0.247 ± 0.003	HPLC
1.4	Thioanisole, ClO_2^-	20,000	$(9.1 + 0.1) \times 10^{-4}$	HPLC
2.0	Br^-, I^-; H_2O_2	10–100	0	UV-vis¶
8.1	Br^-, I^-; ClO_2^-	500	0	UV-vis¶

*Heme and iron were measured as described [Streit BR, DuBois JL (2008) Chemical and steady state kinetic analyses of a heterologously expressed heme dependent chlorite dismutase. *Biochemistry* 47:5271–5280].

†Oxidizable substrates were present in large excess: 1 mM (ABTS) or 10 mM (thioanisole, halides). Aliquots of oxidant were added to reaction mixtures containing enzyme and the oxidizable substrate.

‡Equivalents of oxidant are reported with respect to hemeiron.

§Ratios were computed from three replicate experiments, with the standard deviation reported as the error. The amount of product reported is in excess of the oxidation measured in no-enzyme controls.

¶Experiments were carried out in the presence of monochlorodimedone (MCD) as a colorimetric hypohalide trap.

Table S3. Observed UV-visible bands* for Cld compared with HRP†

Enzyme	CT 1	α	β	CT 2	Soret
Native HRP†	643	≈580	≈530	498	402 (ε =102 mM^{-1} cm^{-1})
HRP† compound I	651, 622 (sh)‡	577	≈525 (sh)		400 (ε = 53.8 mM^{-1} cm^{-1})
HRP† compound II		555	527		420 (ε = 105 mM^{-1} cm^{-1})
Native Cld	650			520	395 ($\varepsilon \sim$ 99.5 mM^{-1} cm^{-1})
Cld compound I§	650	585	525		400 ($\varepsilon \sim$ 72 mM^{-1} cm^{-1})
Cld¶ compound II§		554	525		412 ($\varepsilon \sim$ 94 mM^{-1} cm^{-1})‖
Cld compound II**		555	526		411 ($\varepsilon \sim$ 91 mM^{-1} cm^{-1})‖

*Wavelength values for λ_{max} given in units of nm. CT, charge transfer.

†HRP = horseradish peroxidase; data taken from Dunford HB (1999) *Heme Peroxidases* (Wiley-VCH, New York), pp 141–142. The α- and β-bands of HRP are very weak and observable only in derivatives of the spectral data. Similarly, these bands are not clearly discernable in the spectrum of native Cld above.

‡(sh) = shoulder.

§Prepared with peracetic acid.

¶Tentative assignment of Cld's visible bands is made based on their similarity in energy to those of HRP.

‖Molar absorptivities are computed assuming complete conversion of the starting material to the intermediate. Therefore, these values should be considered lower limits for the actual absorptivities.

**Prepared with ClO^-.